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Kinematic Analysis of the Chemotactic Locomotion of Human Blood Neutrophils: The Effects of Storage at Room Temperature

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KINEMATIC ANALYSIS

OF THE CHEMOTACTIC LOCOMOTION

OF HUMAN BLOOD NEUTROPHILS:

THE EFFECTS OF STORAGE

AT ROOM TEMPERATURE

by

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ABSTRACT

JARRETT LEE BURTON: Kinematic Analysis of the Chemotactic Locomotion of Human Blood Neutrophils: Effects of Storage at Room Temperature. (Under the direction of HARVEY L. BANK)

When human blood neutrophils are isolated for the circulation, they begin to lose chemotactic responsiveness. To characterize this loss of function which occurs during storage, an enhanced videomicroscope system was developed and computer-assisted image analysis was utilized to perform kinematic analysis on neutrophils migrating in an agarose assay system. The locomotor behavior of neutrophils was measured in response to a gradient of chemoattractant (chemotaxis) and in response to an isotropic distribution of chemoattractant (chemokinesis). During chemotaxis, neutrophils migrate at ~20 um/min, make few turns and orient toward a chemoattractant with high accuracy and precision. During chemokinesis, neutrophils migrate at a slower speed, change direction more often and show no directional preference. The vast majority of normal, fresh neutrophils show chemotaxis in response to a gradient, while ~10% of fresh normal cell fail to orient in a gradient. After storage, ~65% of the cells show normal chemotaxis in response to a gradient. The other 35% migrate at 8-9um/min and show no directional preference; they orient randomly. This behavior is quantitatively similar to chemokinesis and suggests that such cells either

cannot detect the gradient or cannot respond to the gradient. A model is proposed which suggests that the ability to orient accurately in a gradient is a function of a structural asymetry within the cytoskeleton and that the loss of this unique structural arrangement by some cells during storage produces slower, non-oriented "chemokinetic" locomotion in response to a gradient.

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CHAPTER I.

GENERAL INTRODUCTION

Human polymorphonuclear leukocytes (neutrophils) are the formed elements of the blood most labile during storage. The ability of neutrophils to carry out normal physiological functions deteriorates very rapidly after isolation (McCullough, et al., 1978; Ferrante, et al., 1980; McCullough, et al., 1980; Lane and Windle, 1981; Glasser, 1982). During storage, the first function to fail is chemotaxis, followed by oxygen metabolism, bacterial killing and phagocytosis (McCullough, et al., 1978; McCullough, et al., 1980; Lane and Windle, 1981; Glasser, 1982). Although stored neutrophils respond poorly to chemotactic stimuli, they are often still capable of phagocytosing and killing bacteria (McCullough, et al., 1978; Lane and Windle, 1978; McCullough, et al., 1980). This leads to an interesting question: If both whole cell locomotion and regional motility (eg. phagocytosis) require controlled activity of the same motile "machinery", why is chemotaxis lost first?

The original specific aims of this research were to 1) identify that particular attribute of neutrophil locomotion which is unique for chemotaxis and 2) characterise the "lesion" which is produced when neutrophils are stored.

I. Why store neutrophils?

Neutrophil transfusions are a valuable adjunct to the treatment of infection in neutropenic or immunocompromised patients, patients with abnormal neutrophil function, neonates and patients who fail to respond to or who are allergic to antibiotics (Cooper, 1982). Infections caused by Gram-negative bacteria cause ~60% of deaths in patients with hematological malignancies (Graw, et al., 1972). A typical transfusion regimen involves 4 transfusions per day (with 3-6 x 10^9 to 10^{10} neutrophils/transfusion) for 7 days (Huestis, 1982). Although new methods of leukapheresis permit the collection of 2-4 x 10^{10} neutrophils from a single donor (Huestis, 1982), the logistical problems of procuring sufficient numbers of transfusible cells can be formidable. Since some neutrophil functions deteriorate rapidly after removal of the cells from the circulation. The ability to store neutrophils, even for the short periods required for transportation and delivery between medical centers would facilitate improved therapy of infected, immunocompromised patients.

II. What is known about the nature of preservation injury?

Circulating neutrophils are fully differentiated cells which mature in the bone marrow, circulate in the blood for a few hours, then diapedese through the capillary walls into the tissues where they continue to function for 4-5 days. The lability of mature neutrophils has been attributed to

their lack of biochemical repair mechanisms.

Previous attempts to develop methods to store neutrophils have been only partially successful. Neutrophils stored at 4°C have poor chemotactic responsiveness (McCullough, et al., 1978; Lane and Windle, 1979, 1980). This "cold-storage" lesion results from the depolymerization of microtubules and disruption of the filamentous actin network at the reduced temperature (Palm, 1981). These effects are not reversible by subsequent incubation at 37°C (McCullough, 1980). Storage at 30-37°C causes a rapid loss of motile function which has been attributed to either the depletion of the glucose supply in the medium (Glasser, 1982; McCullough, et al., 1980) or to autooxidative damage to the cell membrane or the microtubules (Ferrante, et al., 1980).

Currently, storage of neutrophils in plasma, in polyvinyl chloride bags at room temperature (20-22°C) is considered optimal (McCullough, et al., 1980; Lane, et al., 1980; Glasser, 1982). However, neutrophils stored for more than 24 hrs. at 20-22°C show a number of functional alterations summarized in Table I. The <u>in vivo</u> studies have shown that chemotaxis of neutrophils stored under "optimal" conditions begins to deteriorate after only ~8 hrs. storage at 20-22°C (McCullough, et. al., 1983).

TABLE I

FUNCTIONAL DEFECTS IN NEUTROPHILS STORED AT 20-2°c FOR 24 HOURS

FUNCTION	DECREASE	REFERENCES
CHEMOTAXIS	20-50%	Glasser, 1982
CHEMOKINESIS	30%	Lane and Windle, 1981
ATP CONTENT	20%	Lane and Lamkin, 1981
IN VIVO CHEMOTAXIS	30%	McCullough et al., 1983
IN VIVO RECOVERY	50%	McCullough et al., 1983
RECEPTOR AFFINITY	25%	Lane & Lamkin, 1981
EDmax (Chemotaxis)	20%	Richards, et.al., 1982

Casual examination of the data in Table I suggests a correlation between the decreased ATP content and diminished chemotaxis due to the ATP requirement for locomotion (McCullough and Welblen, 1979), yet when the level of ATP in fresh neutrophils was decreased by incubating the cells with 2-deoxyglucose, chemotaxis was not diminished (Lane and Lamkin, 1983). The drop in ATP is correlated with decreased plasma pH and glucose concentration of the suspending plasma (Lane and Lamkin, 1982; McCullough, et. al.,1982). When plasma was supplemented with 15mM sodium bicarbonate (Lane & Lamkin, 1984), pH was maintained better and the extent of the

loss of chemotaxis was reduced. When neutrophils were stored in RPMI 1640 supplemented with 1% human serum albumin, the maintenance of chemotaxis was comparable to that of plasma. (Glasser, et.al, 1985). Therefore the chemotactic defect is not simply a case of cellular "exhaustion" and a more subtle mechanism must exist.

Functions which are not significantly altered by storage at room temperature for 24 hrs. include: oxygen uptake (Glasser, 1982), nitro-blue tetrazolium (NBT) reduction (McCullough, et al., 1980), phagocytosis (McCullough, et al., 1978), bacteriocidal capacity (McCullough, et al., 1978) and the ability to adhere to and spread on a substrate (Palm, et al., 1981). The crucial observation is that not all motile functions are decreased! Even though stored neutrophils do not respond normally to a chemotactic stimulus, the basic motile machinery must be intact for a neutrophil to extend pseudo-pods, phagocytose, or translocate cytoplasmic granules. The major difference between chemotaxis and the other integrated functions is that for chemotaxis to occur the neutrophils must detect a gradient of chemical information and translate that information into sustained, directed locomotion. This suggests that "preservation injury" is selective for a structure or process which is unique for chemotaxis.

III. Possible mechanisms of "preservation injury"

Chemotaxis is an integrated function involving three systems any of which could be involved in defective chemotaxis:

1. Membrane receptors detect the presence and concentration gradient of chemoattractants,
2. Membrane and cytosolic molecules transduce this information into regulatory signals which, in turn, modulate the effector system,
3. The actin-myosin-tubulin-vimentin cytoskeleton converts energy into physical force and motion.

a. Membrane Receptors

Neutrophils have a specific receptor for N-formyl-Methionyl-Leucyl-Phenylalanine (N-fMLP) on their membranes (Williams, et al., 1977) and the affinity of binding parallels the ligand's ability to stimulate directed locomotion of neutrophils (Schiffmann, et al., 1975; Becker, 1979). Neutrophils stored for 24 hrs. at 20-22^{o}C show a decreased affinity of this receptor for its ligand (Lane and Lamkin, 1981, Richards, et.al, 1982). Altered receptor affinity could be responsible for decreased chemotaxis.

Snyderman (1983a,b) reported that receptor exists in two affinity states in isolated membranes: a low affinity state (Kd=20-25nM), which signals oxygen utilization and primary granule (lysosomal) secretion, and a high affinity state (Kd=1-3nM) which signals locomotor activity and

secondary (specific) granule release. The two affinity states may be interconvertible, a shift from one state to another can be induced by changes in the viscosity of the cell membrane (Nelson, et al., 1982; Yuli, et al., 1982; Snydermann, 1983a,b).

An alternative view has been proposed by Marasco and coworkers (1985) They report that on rat neutrophils this receptor exists in a single homogeneous class and shows negative cooperativity, ie., the affinity of unoccupied receptors is decreased as a function of the fraction of occupied receptors (Marasco, et.al., 1985). These authors reported the dissociation rate of N-fMLP from this receptor was afunction of the fractional occupancy of the receptor and suggest that the site-site interactions could be mediated by the release of some "...effector..." from bound receptors which alters the conformation of unoccupied sites.

Binding of N-fMLP to its receptor causes several changes to occur in the membrane including: 1) the aggregation of receptor molecules (Romeo, 1982), 2) an increase in the negative surface charge (Dahlgren and Stendahl, 1982), 3) a decrease in the hydrophobicity of the cell surface (Dahlgren and Stendahl, 1982)and 4) the formation of a complex between the occupied receptors and the Triton-insoluble cytoskeletal proteins (Jesaitis, et.al., 1984). These changes also modulate the affinity of unbound receptors. The processes which are initiated by signals from the different

affinity states of the N-fMLP receptor can be differentially altered: "membrane fluidizing" agents, such as n-pentanol, enhance chemotaxis but do not enhance superoxide anion production nor lysosomal enzyme secretion (Yuli, et al., 1982). When neutrophils were treated with a polyene antibiotic (amphotericin B) which binds to membrane cholesterol, membrane viscosity increased, the N-fMLP receptor was shifted to a low affinity state (~40 nM), and the secretion of lysosomal enzymes was enhanced, while chemotaxis was inhibited (Lohr and Snydermann, 1981).

One possible interpretation of these data is that locomotion and secretion are parallel phenomena rather than steps in a sequence (Becker, 1976; Bass, et al., 1978; Becker, 1979), and storage may selectively affect a step that is critical for chemotaxis.

b. Signal Transduction

Several events occur after the initial ligand-receptor interaction: receptor-mediated endocytosis of ligand-receptor complexes (Niedel, et al., 1979), a rapid depolarization of the neutrophil membrane, followed by 1) a hyperpolarization due to shifts in the cell's permeability to potassium and activation of the sodium-potassium pump (Naccache, et al., 1977), 2) an increase in intracellular calcium due to increased influx coupled with the release of calcium from a membrane associated pool (Hoffstein, 1979;

Naccache, et al., 1979), 3) a transient increase in intracellular cAMP (Naef, 1983)and 4) a rapid turnover of membrane lipids with the release of arachodonic acid and the methylation of some membrane proteins (Hirata and Axelrod, 1980).

A possible sequence of these events begins with the ligand-receptor interaction which induces a conformational change in some membrane or cytoskeletal protein. The cell depolarizes (Seligmann & Gallin, 1983) and bound calcium ions are released into the cytosol in the region of the cell near the occupied receptors (Smolen, et.al., 1982). Schiffmann and coworkers (11983) suggest that when calcium exceeds the critical concentration for a regulatory kinase, the regulatory protein lipomodulin is phosphorylated and deactivated, thus activating phospholipase A_2. Phospholipase A_2 releases arachodonic acid and lysophosphatidyl choline from phosphotidyl choline, which results in increased membrane fluidity. The reduction in the viscosity of the membrane facilitates a passive influx of calcium in the region of the membrane near the occupied receptors (Schiffmann, et al., 1983)

Drugs

The specific temporal sequence in which different functions are activated is not known. However, different cellular functions can be selectively uncoupled from others by the use of various drugs. Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) activates protein kinase C directly

and causes neutrophils to 1) release lysosomal enzymes, 2) increase oxygen metabolism and 3) increase the amount of actin associated with the cytoskeleton without any apparent increase in intracellular calcium (Sha'afi, et al., 1983). Piroxicam is a non-steroidal anti-inflammatory agent which inhibits lipoxygenase, reduces the binding of FMLP to its receptor, reduces the release of membrane calcium and inhibits chemotaxis, with out affecting lysosomal secretion (Edelson, et al., 1982; Scheinberg, et al., 1983). Quinacrine, a lipophilic anti-malaria drug, inhibits membrane depolarization, superoxide production and the degradation of methylated lipids to phosphatidylserine (Tauber, 1983). Mepacrine (a D-L quinacrine mixture) inhibits both chemotaxis and phospholipase A_2 activity (Hirata, et al., 1979).

The signal which is consistently associated with chemotaxis is the change in intracellular calcium. If storage induces a change in the concentration and distribution of intracellular calcium, chemotaxis will probably be effected adversely due to altered cytoskeletal function.

c. Cytoskeletal-Motile System

A likely target for the "lesion" induced by storage would be the part of this effector system which directs locomotion. The cell cortex is composed of actin filaments, associated with an actin binding protein (ABP), which exist in a sol-gel equilibrium state. This reversible transition

is controlled by other proteins, including, gelsolin, which is activated by intracellular calcium. Gelsolin cuts the actin filaments and, thus dissolves the actin gel. Changes in localized cytoplasmic viscosity, due to dissolution and reformation of the actin-ABP gel, causes bulges (pseudopods) to extend outward from the cell. A calcium-independent protein, acumentin, also regulates the length of actin filaments. The calcium-gelsolin complex cuts actin filaments and binds to the "barbed" ends, while acumentin binds the "pointed" ends. When the calcium level drops below 10^{-8}M, gelsolin dissociates from actin. Since the "barbed" ends have a higher affinity for the "pointed" ends than does acumentin, this protein is competitively displaced and actin filaments can lengthen (Hartwig, et al., 1983).

The cortical network also contains myosin. Myosin filaments are activated to crosslink with actin and contract by a calcium activated, calmodulin regulated phosphorylation system. When intracellular calcium exceeds 10^{-6}M, calmodulin activates myosin light-chain kinase resulting in the phosphorylation of myosin, which can then bind to actin filaments. In the presence of magnesium and ATP, this actin-myosin network contracts (Valerius, et al., 1981; Hartwig, et al., 1983).

The following sequence of events has been proposed (Oster, 1984). A local increase in cytoplasmic calcium causes:

a) gelsolin to reduce the structure (and local mass) of the actin-ABP lattice (solation),

b) the local increase in osmotically active particles in the actin-ABP sol, resulting in an influx of water and the extension of a membrane lammelipod (extension),

c) increased precipitation of the myosin filaments, phosphorylation of myosin and crosslinking of phosphorylated myosin to actin (gelaticn),

d) contraction of the lattice in the region of decreased actin structure (contraction).

Since the less structured anterior region, the lamellipod, is in contact with the substrate it pulls the gelled posterior region of the cell forward. The continued extension of lamellipodia establishes new regions of higher structure and waves of contraction (Oster, 1984, Shields & Haston, 1984).

Microtubules may provide support for the less structured regions of the actin network and may organize movement of the cytoplasmic mass. Anderson and coworkers (1983) reported that microtubules assemble preferentially along the axis of movement. Kirschner and Mitchison (1984, 1986) suggest that microtubules exist in a state of "...dynamic instability...". Microtubules are constantly polymerizing and depolymerizing depending upon whether the end of a given tubule is stabilized by the presense of a molecule which prevents depolymerization from that end. If the ends of

microtubules near the leading lamelipod are capped, those tubules will elongate preferentially along the axis of motion. Euteneuer and Schliwa (1985) suggested that such a capping event occurs in migrating neutrophils. They suggest that actin in the advancing lamellipod stabilizes the ends of microtubles and facilitates their elongation. It must be noted that this scheme requires a very high rate of tubulin polymerization (sufficient to elongate a microtubule 5 um with a half-time of 20 sec.) Salmon (1984) reports that microtubules elongate at 0.6 um with a half-time of 20 sec. and suggests that capping one end of a microtubule is not sufficient for stabilization. Thus, the mechanism of elongation along the axis of motion remains to be elucidated.

The intermediate filaments (vimemtin) in neutrophils form a tangled knot in the tail of migrating neutrophil (Davis, et.al., 1982, Hoffstein, et.al., 1982, Parysek, et.al., 1984) and may be involved in stabilizing neutrophil locomotion. Coated vesicles, possibly involved in the endocytosis of bound receptors (Neidel, et.al, 1979) have been found associated with the intermediate filaments (Davis, et.al., 1982, Hoffstein, et.al, 1982). The removal of occupied receptors from the rear of the moving cell could render the rear of the cell less sensitive to stimulation. It is also possible that the microtubules and intermediate filaments could interact in the rear of the cell and thus

influence the direction of locomotion. Storage of neutrophils could alter the organization of these filament system and thus alter chemotaxis.

Phagocytosis

Neutrophils which have been stored for 24 hr. at 20-22°C have essentially normal phagocytic function. The current model of how phagocytosis proceeds (Moore, et al., 1978; Hartwig, et al., 1983; Anderson, et al., 1983; Ryder, et al., 1983; Valerius, et al., 1983) begins with contact of the neutrophil with a phagocytosible particle. This contact induces a receptor mediated localized influx of or release of calcium ions and consequent increased solation and contraction of the cortical actin-myosin network. Actin polymerizes and the cortical cytoplasm and membrane bulge out around the particle. As more membrane receptors contact the particle, a gradient of calcium ions forms, actin polymerizes at the tips of the extending pseudopods and an actin depleted region forms adjacent to the particle. Cytoplasmic granules, which were previously excluded from the cortical region by actin gel, are translocated to the forming vacuole and fuse with it, even before the particle is completely engulfed.

The most likely second messenger for the locomotor machinery is intracellular calcium, specifically, a "membrane-bound" pool of sequestered calcium (Schiffmann, 1981; Hoffstein, 1979; Wilkinson, 1982; Naccache, et al., 1979).

Intracellular calcium is required by several parts of the motile effector system:

1) gelsolin is activated when the intracellular calcium exceeds 10^{-6}M,

2) a calcium-calmodulin complex activates myosin light-chain kinase, which phosphorylates myosin light chains and induces binding and contraction.

3) The local increase in calcium in the immediate vicinity of the membrane may lead to the activation of phospholipase A_2 and a reduction of membrane viscosity, as described previously.

Drugs

The role of calcium in chemotaxis has been probed using several drugs. The inhibition of calmodulin, by trifluperizine, (Smith, et al., 1981), the inhibition of phospholipase A_2 by mepacrine (Hirata, 1979) and the inhibition of lipoxygenase by piroxicam (Scheinberg, et al., 1983) all cause a decrease in chemotaxis.

Other cytoskeleton-specific drugs provide evidence as to which cytoskeletal component(s) is required for chemotaxis. Colchicine, well characterized plant alkaloid, specifically binds to and disrupts polymerized microtubules, prevents polymerization of tubulin into microtubules (Wilkinson, 1982) and inhibits both chemotaxis and the intracellular transport of granules (Becker, 1982). Colchicine also causes

intermediate filaments to collapse into a perinuclear mass.

Malawista, et al. showed that a colchicine-induced increase in cAMP was a consequence of the depolymerization of microtubules rather than its cause (Malawista, et al., 1978). The association of the microtubules with a membrane localized enzyme system (adenyl cyclase) suggests that the microtubules are not merely passive structural elements. Such a microtubule-membrane "connection" suggests that intact microtubular structure does not necessarily indicate normal microtubular function and the dynamic associations of microtubules with other cytoskeletal or membrane components may be essential.

Taxol is an anti-tumor drug which promotes and stabilizes assembly of microtubules, blocks cytokinesis and inhibits the neutrophils' ability to adhere and spread on a substrate (Roberts, et al., 1982). This drug inhibits those functions which require normal microtubule turnover. Again, a defect in one major cytoskeletal component does not necessarily inhibit all motile functions, but an alteration of microtubule function consistently affects chemotaxis.

Cytochalasins inhibit all functions requiring microfilaments, including chemotaxis, by competing for the site of monomer addition on F-actin and by blocking crosslinking (Becker et al., 1972). The relationship of these functions to the microfilaments has not been explained, however a microfilament-membrane "connection" has been demonstrated

(Jesaitis, et.al, 1984) which is disrupted by treatment with dihydrocytochalasin D (Jesaitis, et.al., 1985). Since an intact microfilament system is required for all motile functions (phagocytosis, exocytosis and locomotion), it is highly unlikely that a defect in this system would effect chemotaxis selectively.

d. Locomotor behavior

As noted previously, storage of neutrophils at 20-22°C for 24 hr. does not alter all motile functions. Chemotaxis is a complex function which is usually studied as the aggregate behavior of a population of cells (Wilkinson, 1982). The possibility that neutrophils are heterogeneous in their locomotor behavior and sensitivity to "preservation injury" can only be determined by studying the behavior of individual cells. This approach comprised the majority of this study and yielded several original observations and insights into the nature of chemotaxis of neutrophils.

IV. Experimental plan

The specific aims of these studies were to identify the structure or process in the neutrophil which is a unique attribute of chemotaxis and determine if that attribute was altered by storage at 20-22°C. These studies involved four major stages:

1) development of storage conditons which were similar to current blood-bank practice but which maintained the pH of the suspending medium (Chapter 2),

2) adoption of an assay of chemotaxis which was consistent and reliable (Chapter 2),

3) development of a video system for observing and recording the movement of individual neutrophils (Chapter 3)

4) development and implementation of a rigorous quantitative method for the characterization of the locomotor behavior of neutrophils (Chapters 4,5,6).

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CHAPTER II.

PRELIMINARY DATA AND CONTROLS

In order to focus the research and determine the approach most likely to elucidate the nature of the lesion which develops during storage of neutrophils the following preliminary studies were performed to:

1) develop storage conditons which preserved cell numbers, cell viability and the pH of the medium so that the only experimental variable would be the duration of storage,

2) determine the optimum chemotactic dose of N-fMLP in the agarose assay,

3) measure the production of hydrogen peroxide to determine if oxidative metabolism of stored cells was altered,

4) quantitate the locomotor behavior of individual fresh and stored neutrophils to determine if storage alters how fast the cells migrated, and how accurately and efficiently the cells migrated toward a chemoattractant.

MATERIALS AND METHODS

Isolation of Granulocytes

Fresh human blood was obtained from normal volunteer donors by venipuncture. The blood was drawn with a 21 gauge needle into a plastic syringe containing 1.5 ml citrate-phosphate-dextrose (CPD) anticoagulant (sodium citrate·$2H_2O$, 26.3 gms/L, citric acid, 3.27 gm/L, monobasic sodium phosphate·H_2O, 2.22 gm/L, dextrose 25.5 gm/L) per 8.5 ml of blood.

Erythrocytes were sedimented by mixing 5 volumes of blood with one volume of 6% solution of high molecular weight Dextran (T500, Pharmacia Fine Chemicals, Uppsala, Sweden) in Hank's balanced salt solution without calcium and magnesium (Gibco [Grand Island Biological Co., Grand Island, NY]) containing 25 mM HEPES (Sigma Chemical Co., St. Louis, MO) [HBSS-H] in a 50 ml plastic syringe. The syringe was set upright on its plunger and incubated at 22°C for 30 min. The leukocyte-rich supernatant was then expressed from the syringe into a polypropylene tube, through a butterfly infusion set.

Percoll (Pharmacia Fine Chemicals) was used to separate the leukocyte subpopulations. A stock solution of isotonic Percoll was prepared; 9 vols. of Percoll plus 1 vol. of 10X HBSS (without calcium or magnesium). This 90% Percoll solution was diluted with HBSS-H to 68.4% (=1.096, N_d^{25}=1.3470) and to 54.5% (=1.077, N_d^{25}=1.3449) (Guidicelli,

et al., 1982). A discontinuous gradient was formed in a 15 ml polypropylene tube (25319, Corning Glass Works, Corning, NY); 4 ml of 54.5% Percoll solution layered over 4 ml of 68.4% Percoll, using a 5 ml plastic pipette. The pipette tip was touched to the surface and the 54.5% solution was dispensed very slowly in order to minimize mixing. The leukocyte-rich supernatant was layered on top and the gradients were centrifuged at 450 x g (Sorvall GLC-2) for 20 min. at room temperature. Plasma and upper mononuclear containing layer were collected with a Pasteur pipette and discarded. The granulocyte containing band was collected from the 54.5%/68.4% interface with a Pasteur pipette. These granulocytes were washed with 10 vols. of HBSS-H (450 x g, 10 min.), then the cells were resuspended in 50 ml of HBSS-H, washed (450 x g, 10 min.), then resuspended in HBSS with calcium and magnesium and 25 mM HEPES (HBSS+H), (Gibco).

Enumeration of Cell Types and Viability Assay

Granulocytes and erythrocytes (RBCs) were counted in a Neubauer hemocytometer to determine the number of granulocytes/ml of anticoagulated blood and the number of granulocytes/ml and RBCs/ml in the final washed suspension. The yield of granulocytes/ml of anticoagulated blood and ratio of RBCs to granulocytes (R/W) were calculated.

A sample of the cell suspension was mixed on a slide with an equal volume of 20 uM acridine orange (AO) (Aldrich

Chemical Co., Milwaukee, WI), 20 ug/ml propidium iodide (PI) (Sigma) in HBSS-H (final concentration 10 uM AO and 10 ug/ml PI) incubated at room temperature for 10 min., then examined with a 100x Wild Fluotar oil-immersion objective (NA = 1.25) on a Wild M40 inverted microscope equipped for epiillumination with a Zeiss xenon light source and an FITC filter module.

The nuclei of viable cells showed a green fluorescence due to AO. The segmented nuclei of granulocytes could be readily distinguished from the round nuclei of lymphocytes and the large, lobulated nuclei of monocytes. At least 200 fluorescent cells were classified as either granulocytes or mononuclear cells. "Purity" was expressed as the percentage of granulocytes in the total number viable nucleated cells. Cells with damaged membranes are permeable to propidium iodide. Phenathridinium compounds such as propidium and ethidium have a higher affinity for DNA than does acridine orange and competitively displace acridine orange from the nuclei of damaged cells, therefore their nuclei fluoresce red. Some cells had nuclei showing varying shades of orange/yellow, a metachromatic color shift of the acridine orange indicating DNA denaturation. Such cells were also counted as nonviable. "Viability" was expressed as the ratio of green granulocyte nuclei to the total of green + red (orange/yellow) nuclei. Since the dense bodies of blood platelets were also stained by acridine orange, the residual

platelets were counted and expressed as platelets per 100 granulocytes.

Storage conditions

Initially, isolated neutrophils (2-5 x 10^7/ml) were stored in 5 ml volumes of plasma in tightly capped polypropylene tubes at 20-22°C (Glasser, 1977). After storage the cells were washed twice in 10% CPD in HBSS-H, and resuspended (1 x 10^7/ml) in HBSS+H. These conditions caused sever losses of cell viability (see RESULTS). In all subsequent experiments, cells were stored at a lower concentration (2-5 x 10^6 /ml) in 10 ml of ABO-compatible CPD-A1 plasma (plasma from blood anticoagulated with citrate-phosphate-dextrose containing 275mg adenine) in polyvinyl chloride blood-component storage bags (Delmed, Canton, MA). The bags were stored flat on a wire rack with the label side up.

Chemotaxis

An agarose assay similar to that of Nelson, et.al. (1978) was used to evaluate chemotaxis. A 1% w/v agarose solution was prepared: 2% w/v agarose (SeaKem, High Gelling Temperature, Ultra-Pure, Marine Colloids, Rockland, MN) was dissolved in sterile distilled water at 100°C, then cooled to 56°C and mixed with an equal volume of isothermal 2X Minimum Essential Medium (MEM, Gibco) supplemented with 20% heat-inactivated pooled normal human serum, 50 mM HEPES,

40 mM L-glutamine (Gibco), 0.15% sodium bicarbonate. Six ml volumes of agarose were poured into 60 mm x 15 mm petri dishes (#1007, Falcon Plastics, Oxnard, CA), and allowed to solidify at room temperature. Six sets of three agarose cylinders were cut (3 mm diameter, 5 mm apart, edge to edge) in a radial pattern and the agarose plates were sealed with Parafilm and were warmed in a 37°C/5% CO_2 incubator to equilibrate the pH just prior to use. If any liquid was present in the wells, it was blotted with a small wedge of filter paper. The outer wells received 10 ul of N-formyl-methionyl-leucyl-phenylalanine (N-fMLP) (Calbiochem, La Jolla, CA) in HBSS+H; the middle wells received 10 ul of a suspension of 2.5 x 10^{-7}/ml granulocytes. The inner wells received 10 ul of HBSS+H. The plates were then incubated for 2 hours in a 37°C/5% CO_2 humidified incubator, then examined with a 10x objective using the epifluorescent microscope described above.

The distances which the granulocytes migrated toward the N-fMLP containing well (A, directed migration) and toward the HBSS+H containing well (B, random migration) were measured with a calibrated ocular micrometer (American Optical, Buffalo, NY) mounted in the ocular of the epifluorescent microscope. Results were expressed as millimeters of migration from the edge of the cell containing well. Means and standard deviations of A and B were calculated, as were the chemotactic index (A/B) and chemotactic differential (A-

B) (Nelson, et al., 1978).

The chemotactic response of fresh and stored cells was measured using six different concentrations of N-fMLP (10^{-4} to 10^{-9}M) were tested in each plate. The dose which yielded the highest "A" value was used in all subsequent experiments.

Measurement of Hydrogen Peroxide Production

Oxidative metabolism was monitored by measuring the rate of change in the fluorescence of 2'-7'-dichlorofluorescein diacetate (DCFH-DA) caused by the formation of hydrogen peroxide by neutrophils after stimulation with N-fMLP (Bass, et. al., 1983). A stock solution of DCFH-DA (Eastman Kodak) was made in ethanol at a concentration of 5 mM and stored at -20°C in small aliquots. Percoll seperated neutrophils were suspended in Hank's balanced salt solution containing Ca++ and Mg++ (HBSS+H; Gibco) at a concentration of 2 x 10^7/ml. One hundred microlitres of the cell suspension were put into a cuvette, along with 10 ul of freshly thawed DCFH-DA (final concentration of 50 uM). Sufficient HBSS+H was immediately added to adjust the total volume of solution in each cuvette to 1 ml. The cuvettes were then incubated in the sample chamber for 15 min at 37°C, after which the fluorescence of DCFH-DA was measured with the MPF-2A spectrophotometer. The excitation wavelength was 490 nm (slitwidth of 6 nm) and the emission wavelength was 525 nm (slitwidth of 10 nm). The cells were stimulated by rapidly injecting 0.1 ml of 10^{-6}M

N-fMLP with a Hamilton syringe. The fluorescence for the emitted light was recorded every 5 min for 20 min after it had stabilized from the mixing. The rate of increase in fluorescence (expressed as arbitrary unit per min) is proportional to the amount of hydrogen peroxide produced in the stimulated neutrophils (Bass, et.al., 1982).

Analysis of the locomotion of individual cells

Initial measurements of individual migrating cells were made during chemotaxis. An agarose assay dish was prepared, loaded with cells and reagents and incubated at 37°C for one hr. The motion of cells, at room temperature, was videotaped using an inverted microscope, video camera, VHS video recorder and monitor. The motion of individual fresh and stored cells was recorded for 10 min. During playback, the paths of individual cells were traced onto a sheet of clear plastic which had been attached to the video monitor. The screen was calibrated with a stage micrometer. The length of each cell's path and the start to finish distance were measured. The net angle, relative to the direction toward the chemoattractant, was measured with a protractor. The total distance traveled per min was speed, the start to finish displacement was the velocity, the ratio of velocity to speed was the persistence index of turning and the orientation angle of displacement was relative to 0°, the direction toward the chemoattractant. The definitions of ther

locomotor parameters are illustrated in Fig. 1.

Controls

Freshly isolated neutrophils were incubated in ABO-compatible CPD-A1 plasma, washed twice in 10% CPD in HBSS-H and resuspended in HBSS+H. This "process control" would indicate the effect of manipulation on the locomotor parameters (speed, persistence, orientation and chemotropic index) of chemotaxis.

Chemotaxis of neutrophils from several different donors was measured on different days to determine the extent of donor variability and the reproduciblity of the chemotaxis assay.

Data Analysis

Data are expressed as mean $\pm$ standard deviation. Differences between means were tested for significance by paired or unpaired t-test (95% level) unless stated otherwise.

RESULTS

Recovery, yield and viability of neutrophils

The recovery of neutrophils from anticoagulated blood was 60.5% ± 14.6% (N= 15). This yielded 2.1 x 10^6 ± 0.8 x 10^6 cells per ml of anticoagulated blood. The purity of the final washed suspensions of nucleated cells was 99.0% ± 1.0% neutrophils and 1.0% ± 1.0% mononuclear cells (N = 15). The ratio of erythrocytes to neutrophils was 0.15 ± 0.04 (N = 15). Since no platelets were observed in the final washed suspensions, the residual platelets numbered less that 0.05 per 100 neutrophils (N = 15)

The viability of freshly isolated neutrophils was 95.9% ± 2.7% (N = 15).

Storage conditions

The recommendation that neutrophils not be agitated during storage (Glasser, et. al., 1982) was adopted for this study. The initial storage conditions tested were deleterious to neutrophils. Specifically, storage of neutrophil concentrates in sealed tubes caused a loss of 30% ± 8% of the cells, a decrease in viability from 97% ± 2.0% to 65.3% ± 10.4% and a decrease in plasma pH from 7.2 ± 0.2 to 6.0 ± 0.7 in 24 hr. at 20-22°C (N = 4). The requirement for pH control during storage had been demonstrated for room temperature storage of neutrophils (cf, Chap. 1). Subsequent storage was carried out at lower cell concentrations to prevent substrate

depletion, in standard blood component storage bags. The bags of neutrophils in plasma were stored flat on a wire rack, label side up to minimize pelleting of the cells and to permit maximum gas exchange through the plastic. This modification produced a storage condition where there was no loss of cell numbers or of cell viability during storage. The plasma pH after storage was 7.0 $\pm$ 0.3 (N = 4).

Chemotaxis

The optimum chemotactic dose of N-fMLP was 1 uM for both fresh and stored cells. Stored cells showed less migration toward N-fMLP. The effect of storage on the chemotaxis of neutrophils toward 1uM N-fMLP is shown in Table I. Storage significantly reduces both the directed (A) and non-directed (B) migration. The reduction in the chemotactic differential (A-B) indicates that the directed migration is reduced to a greater extent than non-directed migration.

Hydrogen peroxide production

The data in Table I also shows that stored neutrophils produced peroxides at a rate that was not significantly different from that of fresh cells.

Analysis of the locomotion of individual cells

Table II shows the locomotor parameters of migrating

leukocytes during chemotaxis at 22°C. There was a significant decrease in the velocity and persistence index. The was a significant difference in the orientation angle and an increase in the standard deviation of the orientation angle after storage. The important findings were that, on the average, stored cells did more turning (decreased index) and were less precise (increased SD of orientation angle) in their ability to migrate toward a chemoattractant. It was noted that among the tracings of migrating stored cells there were some paths that were indistinguishable from fresh. This suggests that not all cells are affected by storage to the same extent.

The equipment and techniques for motion analysis were refined and computerized, and the analysis of those results comprises the remainder of this study (Chaps. 4,5,6).

Controls

The computer-assisted method of motion analysis (Burton, et.al., 1986, cf. Chap. 4 for details) was used to determine if the physical manipulation of the cells altered the locomotor parameters. Table III shows that the speed, persistence of locomotion index, and orientation angle of cells which were suspended in plasma then washed were comparable to freshly isolated cells (Chaps. 4,6). The difference in speed between Table II and Table III is due to difference in temperatures between the two experiments. The

data in Table II was collected at 22°C, while that in Table III was collected at 37°C. This indicates that the Q_{10} for chemotactic speed was ~1.9.

Table IV shows data from a series of experiments where the chemotaxis under-agarose of cells from a group of female donors was measured at weekly intervals for one month (Bank, et.al., submitted for publication). These data were analyzed by analysis of variance (F-test) and show that there was no significant variation between samples from a given donor.

DISCUSSION

The purpose of the preliminary studies was verify the methods and control for obvious confounding artifacts.

Collection and purification

The isolation procedure recovered neutrophils from blood with a yield and purity which were comparable to standard techniques (Boyum, 1968). The isolated neutrophils had high viability, and were never exposed:

1) to non-physiological salts such as Hypaque, which alters the cells ability to release its primary granules (Berkow, et.al., 1983),

2) to non-isotonic conditons (such as exposure to hypotonic media to lyse residual red cells) which may specifically alter chemotaxis (Dooley & Takahashi, 1982) or

3) to 0-4^{o}C (kept on ice) which activates neutrophils non-specifically (Keller, et. al., 1984, Haston & Shields, 1985).

The low numbers of residual mononuclear cells and platelets obviates the problem of these cells depleting the plasma of glucose during room temperature storage (Glasser & Fiederlein, 1984).

Storage conditions

Optimizing the conditions of storage has been the major focus of most clinical studies (see Glasser, 1982, for

review), but not ours. The conditions which were adopted circumvented the problems of substrate depletion and decrease in pH, but were not a practical solution to the clinical storage problem due to the relatively large plasma: cells ratio required. The conditions used in these studies maintained cell numbers and viability so that the variable under study was the duration of storage.

Hydrogen peroxide production

The oxidative metabolism of neutrophils is one of the function which should not be altered after 24 hr. of storage. Our data is consistent with that finding indicating that our storage method is comparable to current blood bank procedures.

Analysis of the locomotion of individual cells

The paths of individual stored neutrophils showed wide variability (Table II) as indicated by the increased standard deviations for the PI and orientation angle. There was heterogeneity among the paths for stored cells such that some paths were identical to those of normal cells. This possibility that the effect of storage was selective for certain cells had to be considered.

Controls

The altered chemotaxis which results from storage may

be caused by physical damage which occurs during the handling of the cells. The locomotor parameters of cells which were handled like stored cells, but were not "stored", were measured to test for the effects of the exposure to plasma and centrifugations separate from the effect of storage. The results are comparable to those of fresh cells. Therefore, the alterations of behavior which we measured are not caused by trauma to the cells during handling.

The reliability and consistency of the agarose assay was verified by a series of replicate assays on samples from a group of female donors. The analysis shows no significant difference between the groups of four samples from five different women. The weekly sampling interval also revealed that there was no variation in neutrophils chemotaxis as a function of a given donors menstural cycle (Bank, et.al, submitted for publication).

Conclusion

Chemotaxis is usually discussed as a single function. The behavior of individual cells undergoing chemotaxis can be characterised by a set of parameters which can be measured directly and are expressed in fundamental, physical terms: distances, displacements, angles and time. Several studies had been carried out prior to 1984, when the work described in the following chapters was begun (each chapter refers to the pertinent literature for that specific

problem). At that time, a gap existed in the knowledge concerning chemotaxis because of insufficient understanding of locomotor behavior itself: how are the rate and direction of motion related, how are the turns which a moving cell makes related to how fast the cell is moving, what aspect of the cells behavior changed during storage, how accurately and precisely do neutrophils orient during chemotaxis? How do neutrophils "read" a chemotactic gradient? These questions have not been adequately addressed in the literature.

We, therefore, deemphasized the practical problems of storage and chose to develop methodology with which we could examine the locomotor behavior of individual neutrophils directly, define chemotaxis in terms of fundamental, physical parameters and study the phenomenon of the loss of chemotaxis during storage to test the hypothesis that neutrophils are 1) functionally heterogeneous and 2) are not uniformly affected by storage.

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TABLE I

EFFECT OF STORAGE ON THE CHEMOTAXIS AND HYDROGEN PEROXIDE PRODUCTION OF NEUTROPHILS

	Fresh[a]	Stored
A (mm)	1.39 ± 0.35	0.76 ± 0.50*[b]
B (mm)	0.43 ± 0.26	0.26 ± 0.17*
A-B (mm)	0.96 ± 0.22	0.65 ± 0.32*
H_2O_2 (F/min)	0.70 ± 0.22	0.88 ± 0.48

a. Data reported as mean ± standard deviation

b. * Significantly different from fresh (t-test, $p < 0.05$)

c. Chemotaxis: A = directed migration under agarose toward 1uM N-fMLP, B = random migration, A-B = chemotactic differental, N = 7

d. Hydrogen peroxide production (H_2O_2): rate of increase of fluorescence after stimulation with 100nM N-fMLP, N = 5.

TABLE II

ANALYSIS OF CHEMOTACTIC LOCOMOTION OF FRESH AND STORED NEUTROPHILS

	FRESH	STORED	P
SPEED (microns/min)	6.5 ± 2.3	6.1 ± 2.4	NS
VELOCITY (microns/min)	5.9 ± 2.3	4.3 ± 1.4	>0.05
PI (velocity/speed)	0.91 ± 0.09	0.76 ± 0.21	>0.05
ORIENTATION (degrees)	25.0 ± 16.7	52.1 ± 36.7	>0.05

a.) Data reported as mean ± standard deviation

b.) Note the increased standard deviation of the PI and ORIENTATION after storage.

c.) Analysis performed at 22°C, the cells were tracked for 10 min., fresh = 29 cells, stored = 18 cells

TABLE III

EFFECT OF INCUBATION IN ABO-COMPATIBLE PLASMA ON LOCOMOTOR PARAMETERS OF FRESH NEUTROPHILS

	SPEED (um/min)	PI	ORIENTATION (degrees)
Control	19.6 ± 2.3	0.85 ± 0.15	13.0 ± 37.2
Fresh	20.1 ± 4.2	0.88 ± 0.13	8.0 ± 40.5

a. Data reported as mean ± standard deviation

b. Control: Freshly isolated neutrophils suspended in plasma, incubated for 1 hr. at 20-22°C and washed, N = 54 cells, one donor.

c. Fresh: Data for fresh cells from Chapter 4, N = 307 cells

TABLE IV

CHEMOTACTIC DIFFERENTIAL AND
ANALYSIS OF VARIANCE FOR MULTIPLE
ASSAYS OF CHEMOTAXIS FOR FIVE DIFFERENT DONORS

Donor:	AH	KM1	KM2	BK	KL
Week 1	0.61	0.94	0.70	0.72	0.87
Week 2	0.60	0.96	0.75	0.81	1.44
Week 3	0.62	1.12	1.00	1.18	0.72
Week 4	0.61	0.77	1.11	0.97	0.86

Blood was drawn from five differnt women at weekly intervals for four weeks and the chemotactic response of their neutrophils to 1uM N-fMLP was determined. Data shows the chemotactic differential (A-B) for each sample.

Variation	SS	df	MS	F	p
Between donors	0.35	4	0.088	2.19	>0.05
Among donors	0.6	15	0.04		

a. A-B values were tabulated and analysed by a simple analysis of variance (F ratio).

b. The null hypothesis was: there was not difference between repeated sample from a given donor. The test value for F was 3.06, therefore, the hypothesis was accepted at the 95% confidence level.

FIGURE 1.

LOCOMOTOR PARAMETERS

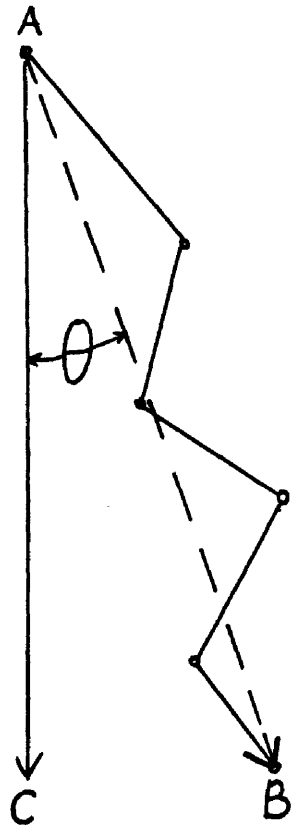
1. SPEED = $\widetilde{AB}$ / min

2. VELOCITY = $\overrightarrow{AB}$ / min

3. PERSISTENCE OF LOCOMOTION INDEX = velocity/speed

4. ORIENTATION ANGLE = θ

5. CHEMOTROPIC INDEX = $\cos\theta$



CHAPTER III.

ANALOG ENHANCEMENT OF VIDEOMICROSCOPE IMAGES

IN PRESS, J. MICROSCOPY, 142 (Pt 3), June, 1986

SUMMARY

A simple, inexpensive technique for enhancing the contrast and resolution of videomicroscope images has been developed. The system has manual controls for gain and pedestal (black level) which permit expansion of low contrast images to the full white-to-black video range. Analog delay-line based circuits are used to sharpen the edges and enhance fine details in the image. These circuits also produce an effective increase in the information content of the image by selectively amplifying low amplitude, high frequency components of the video signal. When live, unstained cells were examined at high magnifications, cytoplasmic structures which were only faintly visible in the unenhanced image become clear. The images of fluorescent objects appear in pseudo-relief, which improves visibility even in the presence of background fluorescense. The system enhances images by performing signal processing functions that otherwise require expensive digital image processing equipment.

INTRODUCTION

The ability of cell biologists to examine, record and analyze highly magnified, low-contrast light microscope images has been extended dramatically by the use of closed circuit video technology. Many subcellular components are separated by distances which are too small to be resolved with visible light. However, such structures still alter the waveform of the illuminating light (Riennitz, 1983). This alteration may be in the form of small phase shifts which are not visible, or as amplitude differences which do not produce sufficient contrast to be visible to the eye or to photographic emulsions. Such information can be partially extracted from an image by optical methods of contrast enhancement, which convert invisible phase information into visible amplitude information. Some of this information still may not produce sufficient contrast to be seen or photographed, however it can be revealed by means of enhanced video techniques (Allen,et. al., 1981a, 1981b, Inuoe, 1981).

Many structures in living cells, which previously could only be resolved by electron microscopy, can now be directly visualized (Allen, et.al., 1981a, 1981b, Inuoe, 1981, Kachar, 1984, Steponkus, et.al., 1984). Digital computer techniques originally developed for analysis of Landsat satellite photographs, have been used to collect spatial, temporal and spectral information from images of cells (Rich & Wampler,

1981, Steponkus, et.al., 1984).. In addition high-sensitivity video cameras, originally developed for night-time surveillance, have been used to visualize minute quantities of fluorescent tracers and to indicate the movement of labeled molecules within cells (Willingham & Pastan, 1978, Neidel, et. al., 1979, Oliver & Berlin, 1982, Herman & Albertini, 1984). The combining of video microscopy with signal processing electronics permits the investigator to extract that information which was previously unavailable due to insufficient image contrast.

Several digital enhancement techniques are being applied to videomicroscope images. These include: 1) the expansion of the contrast range by grey scale manipulation (Allen, et.al., 1981a, Inoue, 1981, Sahota, et.al., 1981), 2) the sharpening of edges (Sahota, et.al., 1981, Walter & Berns, 1981), 3) reduction of random video noise by image summation and averaging (Steponkus,et.al.,1984) and 4) removal of fixed pattern noise by displaying the differences between images (Allen & Allen, 1983). Digital systems perform these functions by converting the video signal into an array of numbers which is then manipulated mathematically. Such digital enhancement methods require digitizers and expensive computer electronics which can perform 10-20 million operations/sec to permit manipulation and continuous observation of the image in real time.

An alternative method of video image processing involves

the use of analog electronics. We have assembled a videomicroscope system which permits manual control and expansion of the gain and contrast ranges of the video signal and uses inexpensive, commercially available analog video circuits which selectively amplify 1) abrupt voltage changes in the signal, thus enhancing edges, and 2) high frequency components of the video signal to correct for the loss of fine details. When this process is applied to an image magnified into the "empty magnification" range, specimen features which could not be visualized due to a lack of sufficient contrast (Reinnitz, 1983) are rendered visible by 1) an effective increase in the signal/noise ratio of the image, 2) a selective sharpening of edges and 3) an increase in the information content due to restoration of low amplitude, high frequencies (fine details). Under favorable optical conditions, this simple analog- enhanced videomicroscope system can detect structures in living cells which cannot be observed directly.

MATERIALS AND METHODS

A. Microscopes

A Wild M-40 inverted microscope (Wild-Heerbrug, Switzerland) was interfaced to a Zeiss II Fl epifluorescence illuminator [Carl Zeiss, FRG](the mounting flange was custom machined to fit the Wild microscope) and a Zeiss trinocular tube head. Recently, an Olympus IMT-2 inverted microscope (Olympus Am., Lake Success, NY, USA) equipped with an Olympus IMT-RFL epifluorescence illuminator was also used. The microscope was bolted to a marble slab which comprised the work-table top. Visco- elastic vibration damping pads (Irving B. Moore, Corp., Cambridge, MA, USA) were used to isolate the table-top from the floor.

The microscopes are equipped with Hoffmann Modulation Contrast optics (Hoffman, 1977). A Wild achromatic-aplanatic condensor (NA = 1.4 oil immersion, 0.95 dry) and an Olympus 100/1.25 oil immersion Planachromat objective were modified for the Modulation Contrast System (the slit aperture and modulator were positioned to use the maximum practical numerical aperture for this objective-condensor combination) [Modulation Optics, Inc., Glenvale, NY, USA]. Wild 10X wide field oculars were used in the trinocular tube head. The microscope image was projected to the video camera by a Zeiss 16X ocular.

B. Video Camera

A Dage-MTI Model 68 camera equipped with a Newvicon tube (Dage-MTI, Michigan City, IN, USA) was modified to permit external user control of gain, pedestal (black) level and signal polarity. The output of this camera is an RS-330, 2:1 interlaced composite signal whose bandwidth was limited to 8 MHz. The Newvicon tube was chosen because although it has slightly lower resolving power than a Vidicon tube it is 20-25x more sensitive. The camera was chosen for its wide range of gain control and its very low noise level.

C. Enhancer/Processor

The video signal was fed to an analog enhancer: Model IV-530 Contour Synthesizer (FOR-A Corp. of Am., West Newton, MA, 6 MHz bandwidth) or an H&V Video Image Enhancer (Vidi-craft Corp., Portland, OR, USA, 5 MHz bandwidth). Contour Synthesis involves the use of a delay-line circuit; the video signal is divided and part of the signal is delayed in time relative to the other. When the signals are recombined, positive voltages are increased, and the abrupt voltage changes which occur at the edges of features are exaggerated.

The H&V Video Image Enhancer performs an "aperture correction" function combined with frequency-selective amplification and delay-line based edge enhancement. A systematic error occurs because the electron beam in the pickup tube is focused to a minute, but finite, spot: this

spot can be considered analogous to an aperture. As this "aperture" scans the illuminated faceplate of the video pick-up tube the abrupt changes in light intensity, which occur at edges in the image, are not accurately converted into voltage changes in the video signal. This occurs because the entire area of the "aperture" is not occluded instantaneously when it crosses an edge. The voltage output represents the average brightness of that area of the image corresponding to the area of the "aperture" at a given moment. A frequency-selective circuit detects and amplifies these voltage changes, restoring the edge sharpness. The H&V enhancer was modified to be side-selective, ie: edges on one side are rendered bright and on the other side are rendered dark (the Contour Synthesizer is inherently side- selective). The visual effect of both devices is analogous to the "shadow-cast" effect seen in Differential Interference Contrast (DIC) microscopy (Galbraith & David, 1976).

A Panasonic WJ-810 time-date generator was used to add a clock and calender to the display. A video processing amplifier (DP-100, 3M Corp., Minicom Div., Camarillo, CA, USA, 5 MHz bandwidth) was used to control total system gain, video black level (pedestal) and to retime and resynchronize the video signal (63.5 usec/line, -0.3V sync level). Multiple levels of gain and pedestal control permit the operator to adjust the contrast of the image over a far wider range than would be possible with a fully automatic camera.

An Hitachi model 302F 30MHz oscilloscope (Hitachi Denshi Am. Ltd., Woodbury, NY, USA) was used to monitor the voltage levels of the different parts of the video signal.

D. Video Recording and Display

The video signal was recorded on VHS format tape with either a GYYR model 2051 time-lapse VCR (GYYR products, Anaheim, CA, USA) or a Panasonic NV-8950 Motion-Analyser VCR (Panasonic, Secaucus, NJ, USA). Images were viewed on a high-resolution Sony PVM-122 12" monochrome monitor (Sony Communications Products, Co., Park Ridge, NJ, USA , 10MHz bandwidth).

E. Model specimens

Fresh human buccal epithelial cells and purified human blood neutrophils were suspended in buffered physiological saline and examined. Bovine endothelial cells were stained with 10 uM acridine orange (AO) and examined with epifluorescent illumination using an FITC filter set.

F. Photography

Photographs of video screens were taken with a Nikon EL 35 mm camera (55 mm, f3.5 Macro lens, 1/4-1/2 sec. exposures) on Kodak 2415 film rated at ISO 250 or Plus-X film rated at ISO 400, developed in Diafine (Acufine, Inc. Chicago, IL, USA). Photographs of live video images utilized the full resolution of the system. Photographs of recorded sequences

are limited to the 300 line resolution of the VHS tape format.

Figure 1. is a schematic diagram of the electronic components of the system.

RESULTS

The optimization/enhancement process involves increasing the gain of the camera until highlights begin to saturate (0.7 V), and then lowering the black level until the edges of the features of interest were completely black (0.05 V). The optimization of signal level was monitored on an oscilloscope to ensure that the entire video contrast range was utilized and that information was not lost due to subjective determination of contrast settings. The time delays available in the enhancers range from 0-300 ns. Typically, a setting of 100-150 nsec (approx. 2 pixel displacment on our 800 line monitor) yielded very sharp edges, without giving the impression of a double image. Edge enhancement was added to the image until video noise (snow) became obtrusive. Gain and pedestal levels were then readjusted.

The sets of photographs were printed so that the contrast of video image was faithfully reproduced. Fig. 2a shows the image of an endothelial cell with automatic gain and black level control and no enhancement, Fig. 2b shows the effect of manual optimization of gain and pedestal level. Fig. 2c shows the added effect of analog enhancement. Figs. 3a and 4a are photographs of images produced by our system using fully

enhancement possible. The appearance of video noise is a function of the video monitor, ie. short persistence monitors show more noise while monitors with longer persistence phosphors, which integrate the random noise over a longer period of time, thus reduce the visibility of the residual noise. The H&V enhancer employs amplitude-selective circuits to limit the amount of noise which may be enhanced. This unit also enhances the higher amplitude portions of the signal selectively, while limiting the enhancement of the low amplitude portions of the signal, thus the visibility and actual amount of enhanced noise is reduced. The Contour Synthesizer does not have such noise reduction circuits; however, its enhancement circuits are inherently less noisy.

DISCUSSION

The system described here uses commercially available components to extract substantial amounts of information from an image at a modest cost. The critical components, the video enhancer and a comparable processing amplifier, can be purchased for ~$3000. Although analog systems are not as versatile as digital systems, which are capable of several modes of contrast enhancement, they can recover information from video images which was not previously visible.

Because of limitations in pixel size, digital processing has been most useful in visualizing extremely highly magnified images. Analog video enhancement has no such limitations and can be utilized on images at lower magnifications to enhance contrast under adverse imaging conditions. For example, we have used the system to visualize neutrophils migrating in a petri dish under a layer of agarose (Burton & Bank, 1985).

The enhancement functions of the system are analogous to two of the most important methods used in digital image processors. One method of digital enhancement involves writing two copies of a video image into a computer's memory, then a constant is added to the X (or Y) address of each picture element (pixel) of one copy of the image. When the two copies are combined, structures in the resulting image have bright or dark edges and a bas-relief (Walter & Berns, 1981) when this process repeated at video frame rates (ie, 30

frames per second). Our analog system generates a comparable effect by delaying one copy of the signal with respect to the other, in time, then recombining them.

The digital analog of "aperture correction" is the process of digital filtering (Steponkus, et.al., 1984). Digital filters operate in the frequency domain (eg., the Fourier transform) or the spatial domain (eg., the Laplacian operator). Both kinds of digital filters manipulate an array of numbers which correspond to the location and amplitude of each pixel of the digitized image and selectively increases or decreases the amplituded(brightness) of pixels that show abrupt changes relative to neighboring pixels. Since abrupt changes often occur at structural edges, the filter operators enhance these changes while suppressing gradual pixel-to-pixel intensity variations (brightness).

The effect of our analog system on resolution and information content is analogous to DIC microscopy. The recombining of two signals separated in time results in the formation of the the derivative of the video signal. The width (duration) of voltage peaks is narrowed, just as the differential interference effect narrows the zeroth order of the diffraction pattern of illuminated objects. The narrower peaks can be slightly closer together in space (light) or time (voltage change) and still be resolved as individual structures (Reinntiz, 1983). Frequency selective amplification is used to amplify weak signals from small variations in

image amplitude. When a stable igh frequency signal is amplified selectively above the random noise level sufficient contrast is gained to render those variations in amplitude visible. Thus the enhanced video image reveals more of the information available in the optical image due to narrowing of the signal peaks and the frequency selective amplification of weak signals.

In the fluorescent image, the highlights are amplified and a black edge is added to each fluorescent structure, thus the contrast of the image is selectively expanded. Since the background fluorescence is homogeneous and featureless, it is not enhanced. In an unenhanced image, weak flourescent spots are often obscured by the background. In our system, these small variations in intensity are enhanced and are rendered more clearly visible. Again, the enhancement process reveals more of the information that was available in the optical image.

Misadjustment of this system can cause distortion of the image and can introduce artifacts. In addition to the problem of the enhancement noise and highlight saturation, excessive amplification and enhancement can cause a double image and the appearance of extra lines at edges. This is analogous to the effect of improper bias compensation in differential interference contrast (DIC) microscopy (cf. Fig 39, Galbraith & David, 1976). Such artifacts can be minimized or avoided by adjusting the video signal using an oscilloscope so that

signal levels can be consistently set for optimum contrast and minimum noise.

This analog system is, therefore, an intermediate solution to the problem of imaging low-contrast microscopic specimens. It offers a dramatic improvement over simple combinations of an automatic video camera, video recorder and monitor; but, avoids the expense of digital processors for those who do not need the versatility of more sophisticated systems. It can be used with any form of optical image enhancement, on fluorescent images and, unlike the situation with some digital image processors, the usefulness of this procedure is not limited to high magnification.

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FIGURE LEGENDS

Figure 1. A block diagram of the electronic components of the analog enhanced videomicroscope system.

Figure 2. a) An image of buccal epithelial cells with automatic gain and black level control, and no enhancement. b) An image of the same cells with manually optimized gain and black level. c) Same as 2b but with enhancement added (Bar = 10 um)

Figure 3 a) An image of a buccal epithelial cell without contrast optimaization or enhancement. b) The same cell with enhancement (150nsec, 0.05 um displacement, bar = 2 um)

Figure 4. a) An image of a neutrophil (unenhanced). b) The optimized image of the same cell with enhancement. (Bar = 2 um)(note: structures which are morphologically similar to mitochondria, arrows)

Figure 5. a) Fluorescent image of bovine endothelial cells stained with AO. b) Optimized, enhanced image of the same cells. (Note the pseudo-relief effect and that some fluorescent objects in 5b are not visible in 5a, arrows, and secondary scattering within the cell illuminates the cell margins, arrowheads, bar = 10 um)

FIGURE 1

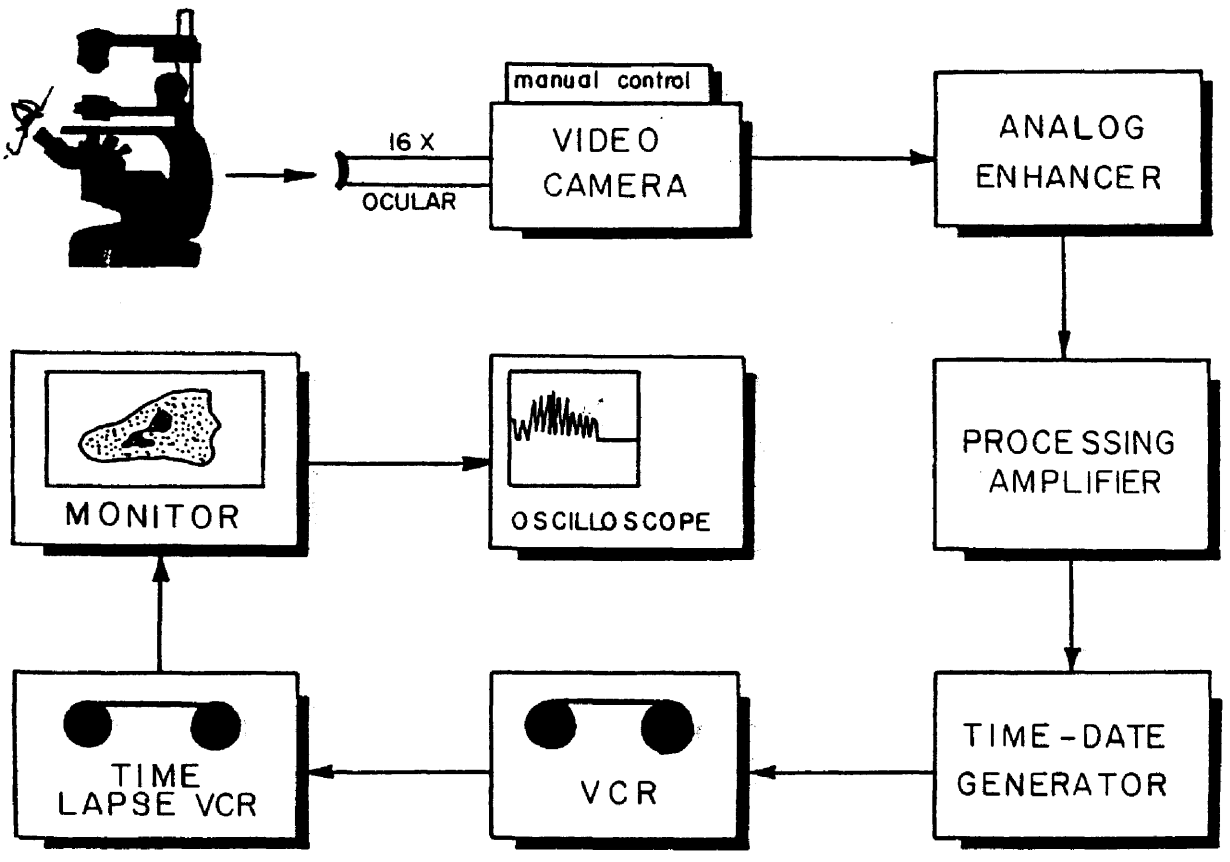

manual control
16 X
OCULAR
VIDEO CAMERA
ANALOG ENHANCER
MONITOR
OSCILLOSCOPE
PROCESSING AMPLIFIER
TIME LAPSE VCR
VCR
TIME-DATE GENERATOR

FIGURE 2a-c

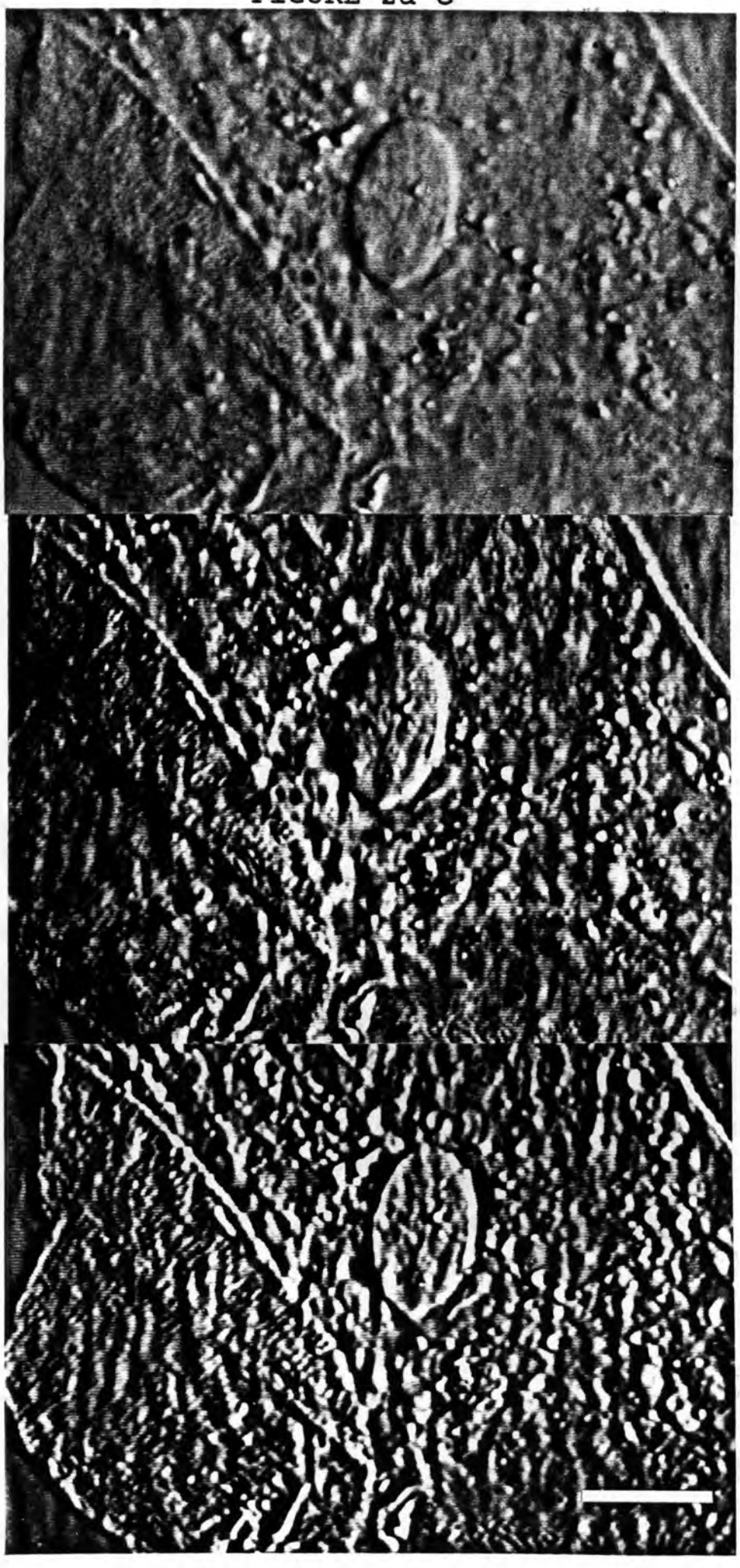


FIGURE 3a-b

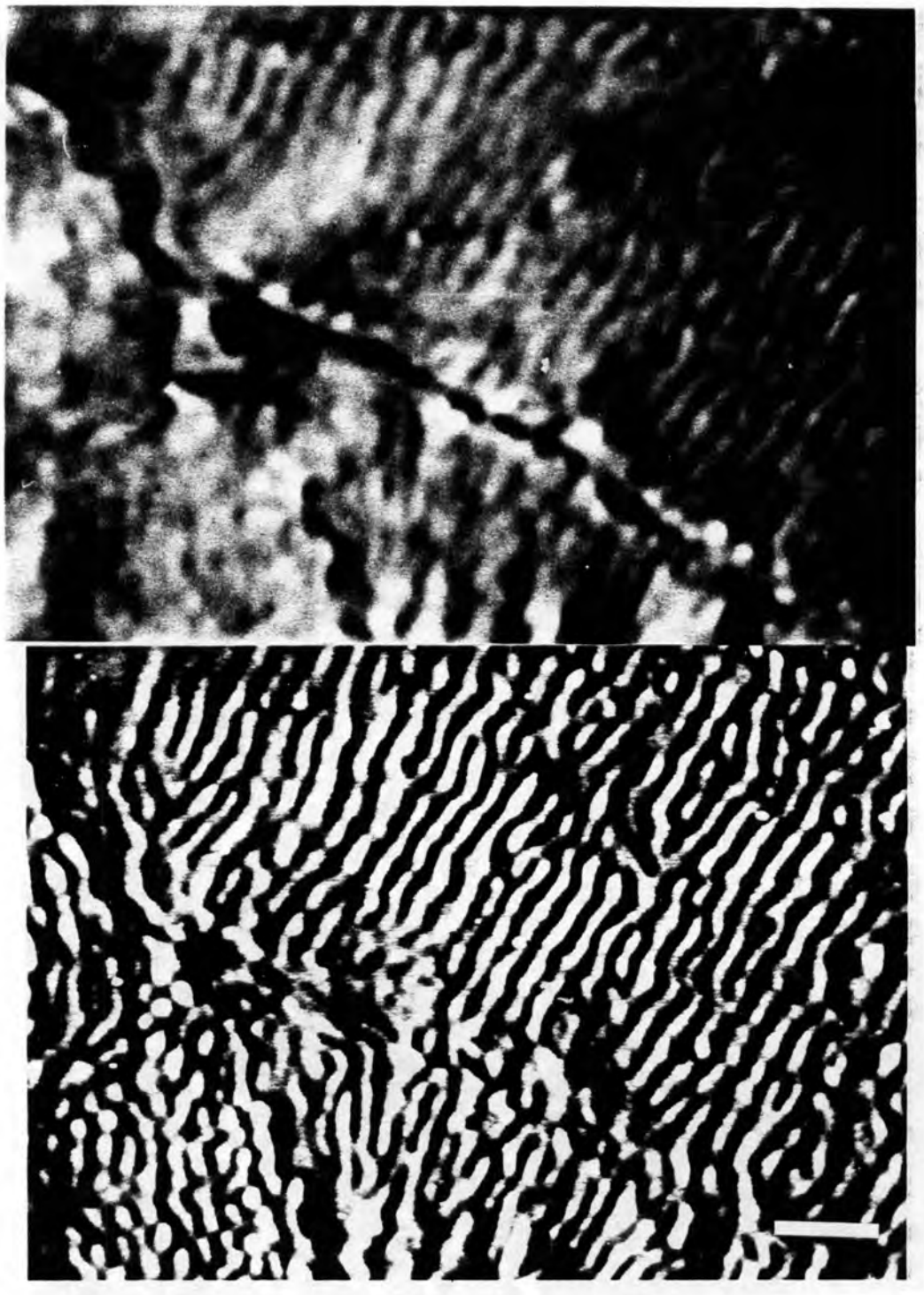


FIGURE 4a-b

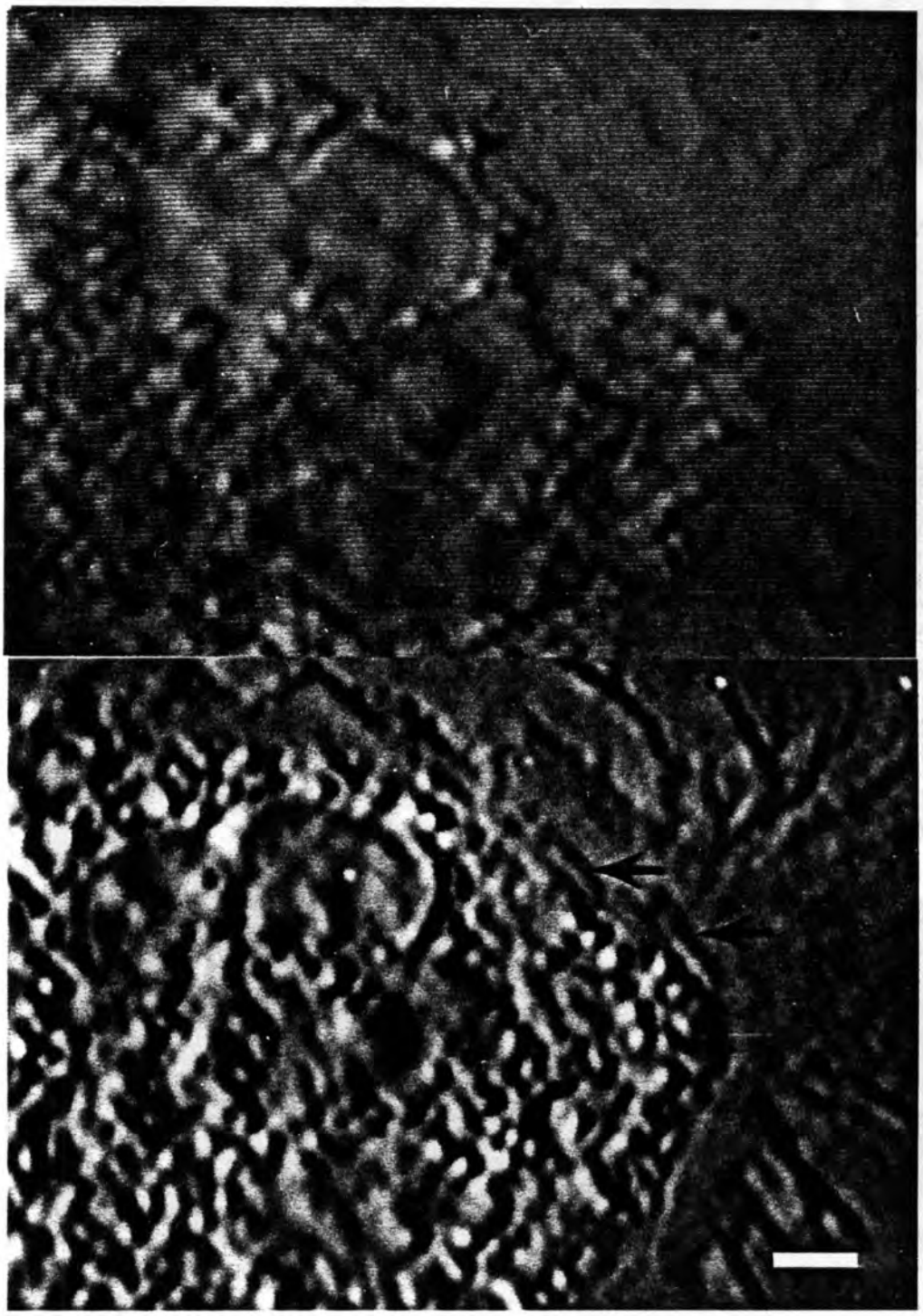
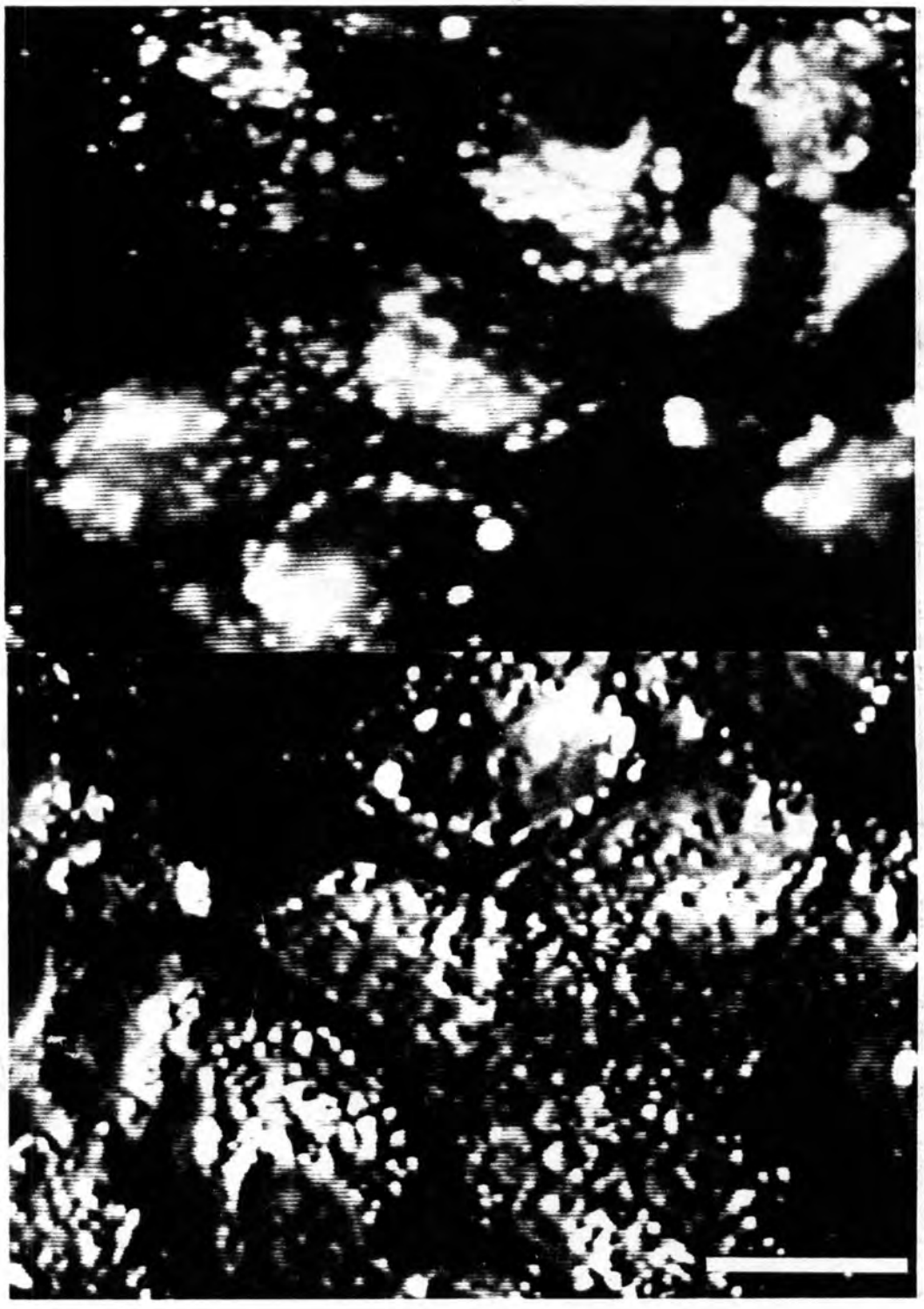


FIGURE 5a-b



CHAPTER IV.

VIDEO ANALYSIS OF CHEMOTACTIC LOCOMOTION OF STORED HUMAN POLYMORPHONUCLEAR LEUKOCYTES

(In press, Cell Motility and the Cytoskeleton)

ABSTRACT

Previous studies of the storage of polymorphonuclear leukocytes (PMNs) storage have used an empirical approach to define "optimal" conditions. To date, no storage conditions have been described which satisfactorily preserve the chemotactic function of PMNs beyond 24 hr. In an effort to define the precise nature of the storage lesion, we studied the chemotactic locomotion of freshly isolated PMNs and PMNs which had been suspended in CPD-A1 plasma and stored in PVC bags at 20-22°C for 24 hr. We used time-lapse video recording and computer image analysis to quantitate the motion of PMNs migrating under agarose. The positions of individual motile cells were traced at 1 min intervals for 5 min. The following parameters were used to quantitate migration: 1) speed (distance/min), 2) persistence of locomotion index (velocity/speed), 3) orientation angle (the angle of the vector describing the net displacement of a cell relative to a direct line towards the chemoattractant) and 4) chemotropic index (cosine of the orientation angle). After 24 hr of storage, the following changes were observed: 1) fewer cells migrated, 2) the speed of migrating cells was reduced by 25%, 3) the persistence of locomotion index decreased by 7%, which indicates that migrating cells made slightly more/wider turns, and 4) the chemotropic index was decreased by 30% which indicates that migrating cells were less accurate in their orientation towards the chemoat-

tractant. Apparently, the storage of PMNs selectively impairs some cells ability to orient accurately in a chemotactic gradient, and changes the distribution of these locomotor parameters within the population.

INTRODUCTION

A satisfactory method for the storage of polymorphonuclear leukocytes (PMNs) for transfusion has not been developed. Despite a series of biochemical studies designed to identify the mechanism of this loss of chemotactic function (McCullough, et. al., 1979, Lane, et.al., 1984, Lane, et.al., 1985a, 1985b.), and thereby design more effective storage conditions, current methods of liquid storage do not preserve chemotaxis for more than 24 hr (McCullough, et.al., 1980, Glasser, 1982, Glasser, et.al., 1983). Conditions which minimize the deleterious effects of short-term storage on PMN chemotaxis have been developed (Glasser, et. al., 1983). These involve the collection of blood in CPD, CPD-A1 or ACD by single unit phebotomy or centrifugal apheresis and storage in PVC bags in plasma, at 20-22^{o}C, without agitation.

Although, PMNs stored under these "optimal" conditions can phagocytose and kill bacteria, <u>in vitro</u>, even after 72 hr of storage (Glasser, 1982), their <u>in vitro</u> chemotaxis is impaired after only 24 hrs (McCullough, et.al., 1980). When such stored cells were transfused, fewer cells migrate into sites of infection or inflammation. Indeed, <u>in vivo</u> chemotaxis begins to deteriorate after only 8 hrs of storage at 20-22^{o}C (McCullough, et. al., 1983). The critical questions remain: what happens to the cells during storage and why is chemotaxis impaired selectively.

Chemotaxis is a complex reaction which can be characterized by the mechanics of the motion, which include how fast the cells move, how often they turn and how accurately they orient toward a chemoattractant. In order to determine which components of chemotaxis are affected by storage and, thereby, extend our understanding of PMN chemotaxis, the locomotion of fresh PMNs and PMNs stored for 24 hr was evaluated in an agarose assay. We recorded the motion of the cells using time-lapse video recording, then used computer-assisted image analysis to quantitate the locomotor parameters.

MATERIALS & METHODS

Collection of Cells

Blood was collected from normal, healthy volunteers by venipuncture. Informed consent was obtained from donors according to the guidelines of the MUSC Committee for the Protection of Human Subjects. Citrate-phosphate-dextrose (CPD) was used as an anticoagulant. The erythrocytes were sedimented with 1% Dextran (T-500, Sigma, St. Louis, MO), for 30 min at room temperature. The leukocyte-rich plasma was underlayered with 55% (ρ = 1.077) and 68% (ρ = 1.097) Percoll (Pharmacia, Uppsala, Sweden) (Guidicelli, et.al., 1982) which was prepared in Hepes-buffered Hank's Balanced Salt Solution, without calcium and magnesium [HBSS-] (GIBCO, Grand Is., NY). Percoll has been shown to facilitate isolation of PMNs which have not been non-specifically activated (Roberts, et.al., 1984, 1985). The gradients were centrifuged at 450 x g for 20 min at room temperature. The PMNs were collected from the 55%/68% interface and washed once in HBSS-, once in Hepes-buffered Hank's balanced salt solution with calcium and magnesium [HBSS+] and resuspended in HBSS+ at 1 x 10^7 cells/ml.

Storage

PMNs were resuspended in ABO-compatible CPD-A1 plasma (generously supplied by the Dept. of Laboratory Medicine, MUSC) at 2-6 x 10^6/ml and stored in polyvinyl chloride blood component bags (150 ml vol., lot 211140, Delmed, Canton, MA)

at 20-22°C without agitation for 24 hrs. After storage, the cells were washed twice in HBSS- containing 10% CPD, and resuspended in HBSS+ at 1 x 10^7 cells/ml.

Chemotaxis

Chemotaxis was assayed with a modified agarose system (Nelson, et. al., 1978). Briefly, a 1% agarose gel (SeaKem, HGT, FMC Bioproducts, Rockland, ME, lot 62040) was prepared in Minimal Essential Medium with Earle's salts (GIBCO, lot 20N4024), containing 25mM HEPES (Sigma), 15mM sodium bicarbonate (Fisher, Norcross, GA), 2mM L-glutamine (GIBCO) and 10% heat-inactivated human serum (serum from 16 normal donors was pooled, aliquoted and stored at -20°C). Six ml volumes of gel were poured into 15 x 60mm plastic petri dishes (Falcon Plastics, Oxnard, CA) and allowed to solidify. Holes were cut in the gel in a radial pattern (3mm diameter wells, spaced 5 mm apart and arranged in groups of three) and the agarose plugs were removed to form wells. Ten microliters of 1uM N-formyl-methionyl-leucyl-phenylalanine (N-fMLP, Calbiochem, LaJolla, CA) was placed in the outer well. Preliminary experiments showed that 1 uM fMLP elicited a maximum chemotactic response from both fresh and stored cells in our system. The middle well received 10 ul of cell suspension (1 x 10^7/ml) and the innermost well received 10 ul of HBSS+. The dishes were incubated at 37°C in a 5% CO_2 atmosphere for one hour, to allow sufficient time for the gradient to form and to permit migration of the

cells to begin. The dishes were then removed from the incubator and sealed with Parafilm, to prevent evaporation.

Video Analysis

An inverted microscope (Wild M-40, Wild-Heerbrug, Switzerland) equipped with Hoffmann Modulation Contrast Optics (Modulation Optics, Glenvale, NY), and a heated (37°) specimen stage (Cambion, Cambridge, MA) was used.

The time-lapse video system consisted of a Dage-MTI model 68 camera (Dage-MTI, Michigan City, IN), a FOR-A model IV-530 Contour Synthesizer (FOR-A Ltd, W. Newton, MA), which enhanced the contrast of the image and facilitated localization of the centers of the PMNs, a 3M model D-100 Processing Amplifier (3M, Minicom Div., Camarillo, CA), which controlled the overall system gain, pedestal and synchronization levels, a Panasonic model WJ-810 time-date generator (National Panasonic, Secaucus, NJ), a GYYR model 2051 time-lapse video recorder (GYYR, Anahiem, CA)and a Sony model PVM-122 monitor (Sony USA, Atlanta, GA). A complete description of the analog-enhanced video system has been published elsewhere (Burton, et.al., 1986a).

The sealed chemotaxis dish was arranged on the stage such that the cell containing well was just off the top of the video screen. Such a placement of the video field permitted observation of those cells which were capable of migrating in response to N-fMLP, but also of any cell which migrated out of the well and into the video field, whether

that motion was directed or not. The video field measured 330um x 250 um (Fig 1) and the image of a typical PMN measured ~1 x ~1.5 cm. Videorecordings were made at 5 frames/sec.

Computer Analysis

A sheet of transparent plastic was placed over the face of the video monitor and the videotapes were played in a freeze- frame mode. In each of 5 pairs of experiments, the centers of 20-30 randomly selected cells were marked on the overlays; the recording was advanced 100 frames (20 sec), and the cells were followed for a period corresponding to 5 min of real time. The error introduced by subjectively determining the center of a cell was negligible compared to the movement of the cell during the interval between measurements.

The plastic overlays were then transferred to the digitizer pad of a Videoplan Image Analysis Computer (Carl Zeiss, Oberkochen, FRG), and points on the paths of individual cells were digitized to record the position of each cell at 1 min. intervals. The digitizer was calibrated by a scalar standard and the direction toward the chemoattractant was defined as 0^{o}.

We selected those parameters which rigorously describe tactic behavior. These parameters are the scalar speed (distance/time), and the vectorial parameter of velocity (displacement/time) and direction (Allan, et. al., 1978,

Keller, 1983, Maher, et. al., 1984, Zigmond, et. al., 1981). In any tactic response the reference direction (0^{o}) is defined by the azimuth toward the "source" of the stimulus, thus the direction of locomotion of an object becomes an orientation relative to the reference direction (Bean, 1979, Bell, et.al., 1982, Ross,1985). The presence of a chemoattractant alters the turning behavior of PMNs (Keller, et.al., 1985) and other kinds of motile cells (Bell, et.al., 1982). The ratio of the vectorial displacement (velocity) to the scalar distance (speed) is a dimensionless indicator of turning behavior called persistence of locomotion (Allan, et.al., 1978, Keller, 1983).

Other commonly used measures of locomotor behavior can be derived from these three independent measures; for example, the McCutcheon or Chemotropism index (Allan, et. al., 1978, Bultman, et. al., 1983) has been defined as the ratio of the distance migrated toward the source to the total distance migrated. Thus, the distance migrated toward the source is equal to the net displacement multiplied by the cosine of the orientation angle. If a cell moves directly toward the source (orientation angle = 0^{o}, cosine of angle = 1) and makes no turns (persistence index = 1), then the McCutcheon index will be 1. If a cell makes turns or has a net displacement at an angle other than 0^{o}, then the McCutcheon Index is <1 because the distance migrated toward the source will be equal to the total distance

migrated multiplied by the product of the persistence index and the cosine of the orientation. The McCutcheon Index is, therefore, a composite value equal to the product of the persistence of locomotion and the cosine of the orientation angle.

The three parameters of net displacement/ time (velocity), total distance/ time (speed) and orientation are sufficient to fully characterize the tactic response of individual PMNs and we use the velocity/ speed ratio (persistence of locomotion index) and the cosine of the orientation angle (called the chemotropic index) to differentiate turning behavior from accuracy of orientation.

The mean, standard deviation and standard error were calculated for the: 1) total distance traveled in micrometers, 2) speed (distance/min) in micrometers/ min, 3) net displacement in micrometers, 4) persistence of locomotion index (net displacement/total distance), 5) velocity (persistence of locomotion index x speed) in micrometers/min, 6) orientation angle (angular component of the velocity vector in degrees) and 7) chemotropic index (cosine of the orientation angle) for each cell path. These data were collected from 5 pairs of experiments (fresh [307 cells] vs. stored [239 cells]) using 5 different donors. The significance of the differences between parameters for fresh vs. stored cells was tested using an unpaired t-test (95% confidence limit).

RESULTS

The rate of formation of the N-fMLP gradient in the under-agarose assay as well as the stability and shape of the N-fMLP gradient have been characterized previously (Lauffenburger & Zigmond, 1981, Dahlgren, et. al., 1984) When cells and N-fMLP are placed in their respective wells at the same time, a stable linear gradient forms in one hour (Dahlgren, et.al., 1984).

In our system, the diffusing front of N-fMLP is not planar; however the extent of curvature can be calculated. The radius of the circle around the chemoattractant containing well which bisects the video field is 6.325 mm, its circumference is 39.7 mm. Since the video field subtends a ~3° arc (331 um) of that circumference, its 330um width also defines a ~3° chord of that circumference. The chord/arc ratio = 0.997; thus, the curved diffusing front deviates from planar by ~0.3% in the center of the video field (Fig 1).

The mean speed of fresh cells undergoing chemotaxis was 21.15 ± 4.18 um/min (Table I). This value falls within the 17 to 30 um/min range reported by other authors (Allan, et. al., 1978, Bultmann, et. al., 1983, Keller, 1983, Maher, et.al., 1984). Likewise, the persistence of locomotion index of fresh cells (0.88 ± 0.13) is comparable to other reports which use a net displacement/total distance (Allan, et. al., 1978) or speed/velocity ratio (Keller, et.al., 1983,

1985). The orientation angle (8.0 ± 40.5°) shows that 68% of the cells had net displacements in an 80° sector toward the chemoattractant(Table 1). This is comparable to the findings of Zigmond (Zigmond, et. al., 1981) who reported that 75% of fresh motile PMNs oriented and migrated in a 90° sector toward a chemoattractant.

When compared to fresh cells (Table 1), fewer stored cells were available for tracking during the observation period (239 vs. 307). For those stored cells which migrated into the video field, we measured 1) a 24% decrease in speed, 2) a 7% decrease in persistence of locomotion, 3) a significant difference between the average orientation angle, 4.) a 60% increase in the standard deviation (SD) of the orientation angle, 5) a 30% decrease in the chemotropic index, and 6) a 70% increase in the SD of the chemotropic index. The slight decrease in the persistence index indicates that the cells were making more/wider turns. The increase in the standard deviations of the orientation angles and chemotropic indices indicates that 68% of the stored cells oriented in a 130° sector [compared to an 80° sector for fresh cells] toward the chemoattractant source; that is, there was a significantly greater number of stored cells whose net displacement was oriented away from the chemoattractant.

Figs 2-5 are the frequency histograms for each of the locomotor parameters. The data for stored cells was

normalized by multiplying the value of each group by 307/239 to adjust for the fewer number of motile cells after storage. Fig. 2 shows that the speed of the population of stored cells is lower due to the presence of some cells which moved at subnormal speeds. The histogram of the persistence of locomotion index (Fig. 3) shows that the decrease in that parameter is due to the presence of fewer highly persistent cells after storage, ie; more of the stored cells made more/wider turns. The histogram of the orientation angles (Fig. 4) shows that many stored cells are oriented at angles $>90^{o}$, thus, many stored cells were migrating away from the chemoattractant. The histogram of the chemotropic index (Fig. 5) clearly shows that some stored cells lost the ability to orient in a chemotactic gradient.

DISCUSSION

Most assays of chemotaxis measure the average performance of the entire population of PMNs. Even if the expression of a behavior is normally distributed in the population, the behavior of an individual cannot be predicted from the average behavior of the population. Population data is, therefore, necessary but not sufficient for the complete characterization of behavior.

Our data clearly shows that most motile fresh PMNs moved rapidly toward a chemoattractant making few, relatively narrow turns along the way. A very small percentage of these motile fresh cells moved at a slower speed (Fig. 3), tended to make more turns (lower persistence index) [Fig. 4] and were less accurate in their orientation in the chemotactic gradient (low chemotropic index) [Fig. 4-5]. Correlation analysis of the data for fresh cells has revealed that neither the accuracy of orientation nor the persistence of locomotion index of a given cell can be predicted from its speed. This suggests that a direct correlation between these locomotor parameters does not exist in fresh cells (Burton, et.al., 1986b).

After storage for 24 hr., fewer PMNs migrated out of the wells and into the video field. This is consistent with the earlier observation by Glasser (Glasser, et. al., 1977) that fewer stored PMNs migrated in their agarose assay. A larger percentage of the motile stored population moved at a

slower speed, made more/wider turns and were less accurate in their orientation. The net effect of storage was to decrease the total number of cells which were capable of migrating under agarose and to decrease the average distance which stored PMNs can migrate under agarose (McCullough, et. al., 1980).

Therefore these studies have shown that:

1) the agarose system can be used to make reliable measurements of the locomotor parameters of individual moving cells,

2) the McCutcheon (Chemotropism) Index can be separated into two components, persistence (the velocity:speed ratio) and the cosine of the net orientation angle, which can be measured independently,

3) storage of PMNs under the conditions described alters their chemotactic response such that the population is slower due to an apparent change in the distribution of the <u>speed</u> parameter rather than a slowing of the entire population,

4) Stored PMNs do not show dramatically increased sizes/numbers of turns,

5) stored PMNs may still orient accurately, but the precision of orientation is reduced, ie: more cells orient at angles away from the source.

Our results are consistent with data from other investigators (Harvath, et. al., 1982, Elgefors & Olling,

1984, Howard, 1982) indicating the existence of functional subsets of normal PMNs. For example, Harvath et.al (Harvath, et. al., 1982) have shown that there is a subset of fresh PMNS which binds N-fMLP normally, but which does not migrate. Elgefors & Olling (Elgefors & Olling, 1984,) identified three subclasses of PMNs by their motility on slides in serum. They described rapidly moving (55%), slow moving (20%) and non-moving (25%) cells in samples from normal donors. They alsc observed that several clinical conditions (eg., bacterial or viral infections) caused a shift in the proportions of cells in the three categories such that more cells were slow moving or non-moving. Our data indicates that storage may induce a similar change causing more PMNs to become slow moving or non-motile, thereby reducing the total number of chemotactically responsive cells.

Howard (Howard, 1982) has identified two subsets of PMNs by their speed and the size of their turn angles during random locomotion and showed that "fast" PMNs tended to make narrower turns than "slow" PMNS. The effect of storage could be a shift of PMNs out of a subset characterized by fast, persistent, accurate migration and into a subset characterized by slower speed, decreased persistence and decreased ability to orient in a chemotactic gradient. Such a population shift is consistent with change in the <u>shapes</u> of the histograms (Burton, et.al., 1985) of the different

locomotor parameters after storage (Figs. 2,4,5). Testing this hypothesis of shifts between pre-existing subpopulations will require the collection of a larger database and determination whether a multi-population model can be developed which fits that data.

Several investigators have shown that selective damage to a specific cytoskeletal structure or biochemical event in PMNs can result in the impairment of a single locomotor parameter. Allan and Wilkinson (Allan, et. al., 1978) showed that PMNs treated with colchicine oriented accurately (were able to locate and phagocytose Candidia spores), but made more/wider turns (lower persistence index) and had slightly decreased speed. These results imply that microtubules are involved in both the movement of the cell and in the persistence of locomotion but not in the orientation of the cell. Valerius (Valerius, 1985) reported that patients with Immotile Cilia Syndrome have defective PMN chemotaxis due to impaired ability to orient in a chemotactic gradient, this defect correlates with an observed decreased number of centriole-related microtubules. Bultmann (Bultmann, et. al.,1983, 1984) studied the chemotactic locomotor parameters of PMNs after infection with ECHO 9 virus, and found that the accuracy of orientation was impaired, while speed (track velocity) was not altered. This effect was attributed to an alteration of the membrane caused by the presence of viral capsid proteins which interfered with that aspect of

receptor function which signaled the direction of the chemotactic gradient but did not interfere with the activation of locomotion. If storage causes an alteration of a specific process or structure, which affects PMNs randomly during storage, then our data shows that only a portion of the population of stored PMNs show impaired chemotaxis. The remainder are not altered after 24 hr.

These two hypotheses may not be mutually exclusive and studies are in progress to test the major assumptions of both hypothesis:

1) that there are subsets of normal PMNs which are characterized by their locomotor parameters and that storage causes cells to switch between pre-existing functional subsets, or

2) that there is a correlation between the individual locomotor parameters and specific cellular structures, thus storage damage is manifest as a lesion to a specific structure.

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TABLE.I VIDEO ANALYSIS OF CHEMOTACTIC LOCOMOTION

Parameter	Fresh	Stored[+]
Speed (um/min)	20.12±4.18	15.37±7.7*
Persistence of Locomotion Index	0.88±0.13	0.82±0.19*
Orientation Angle (degrees)	8.05±40.5	-5.2±65.16*
Chemotropic Index	0.79±0.34	0.55±0.59*

[+]PMNs stored in ABO-compatible CPD-A1 plasma at 20-22° C for 24 hr. Mean ± SD for 5 pairs of experiments are given.

*Means are significantly different at the 95% confidence level when tested by unpaired t-test (two-sided hypothesis, df= 307 for fresh, 239 for stored).

Note: increase in SD of the Orientation Angle for stored cells indicates a decrease in accuracy compared to fresh cells.

FIGURE LEGENDS

Fig. 1 Schematic drawing showing the position of the video field and the amount that the curved diffusing front of N-fMLP deviates from linear at the center of the video field. This drawing is not to scale.

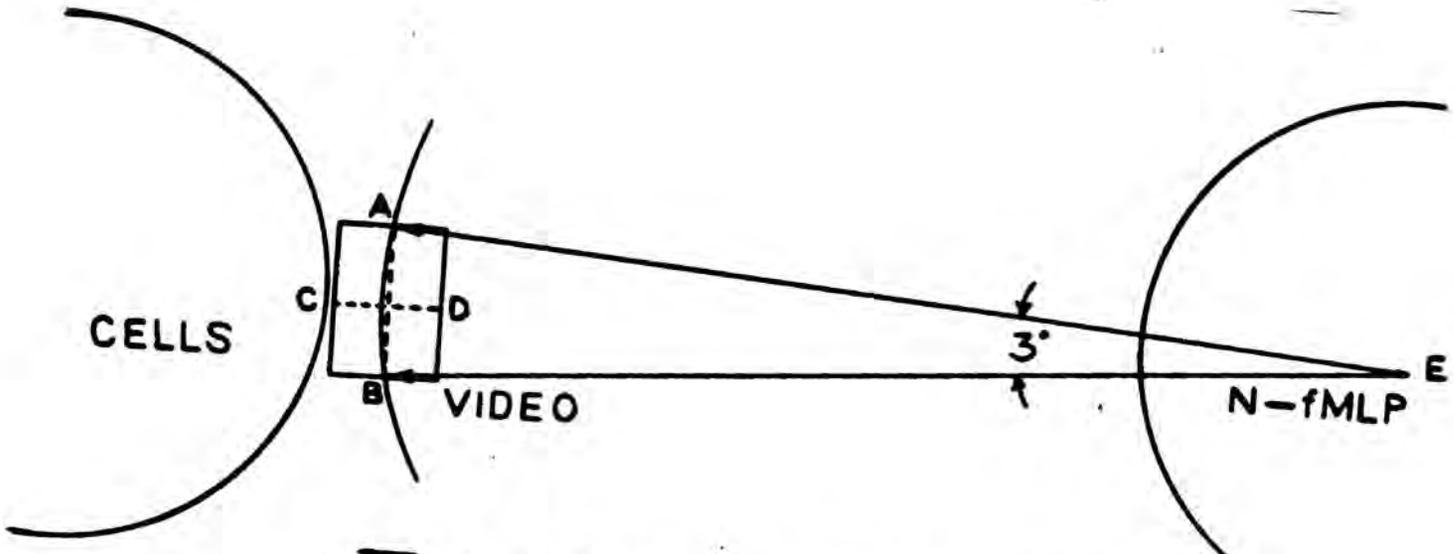
Fig. 2 Histogram of Speed of fresh and stored PMNs. Plot of stored cells was normalized to correct for the lesser number of stored cells analyzed by multiplying the number of stored cells in each interval shown by 307/239. F = fresh PMNs, S = stored PMNs. Note the shift of the frequency distribution to the left indicates that more of the stored PMNs migrated at slower speeds.

Fig. 3 Histogram of Persistence of Locomotion Index of fresh and stored PMNs. Plot of stored cells was normalized to correct for the lesser number of stored cells. F = fresh PMNs, S = stored PMNs. Note that more of the stored PMNs had a slightly lower persistence of locomotion.

Fig. 4 Histogram of the absolute values of the Orientation Angle of fresh and stored PMNs. Plot of stored cells was normalized to correct for the lesser number of stored cells. F = fresh PMNs, S = stored PMNs. Note that more of the stored PMNs had orientation angles > 90^{o}.

Fig. 5 Histogram of Chemotropic Index of fresh and stored PMNs. Plot of stored cells was normalized to correct for the lesser number of stored cells. F = fresh PMNs, S = stored PMNs. Note that more of the stored PMNs had negative chemotropic indices, ie; they migrated away from the chemoattractant.

FIGURE 1



$\overline{AB}$ = 330 um $\quad \widehat{AB}$ = 331 um
$\overline{AE}$ = 6.325 mm $\quad \overline{CD}$ = 250 um
$1 - (\overline{AB}/\widehat{AB}) \times 100 = 0.3\%$

NOT TO SCALE

FIGURE 2

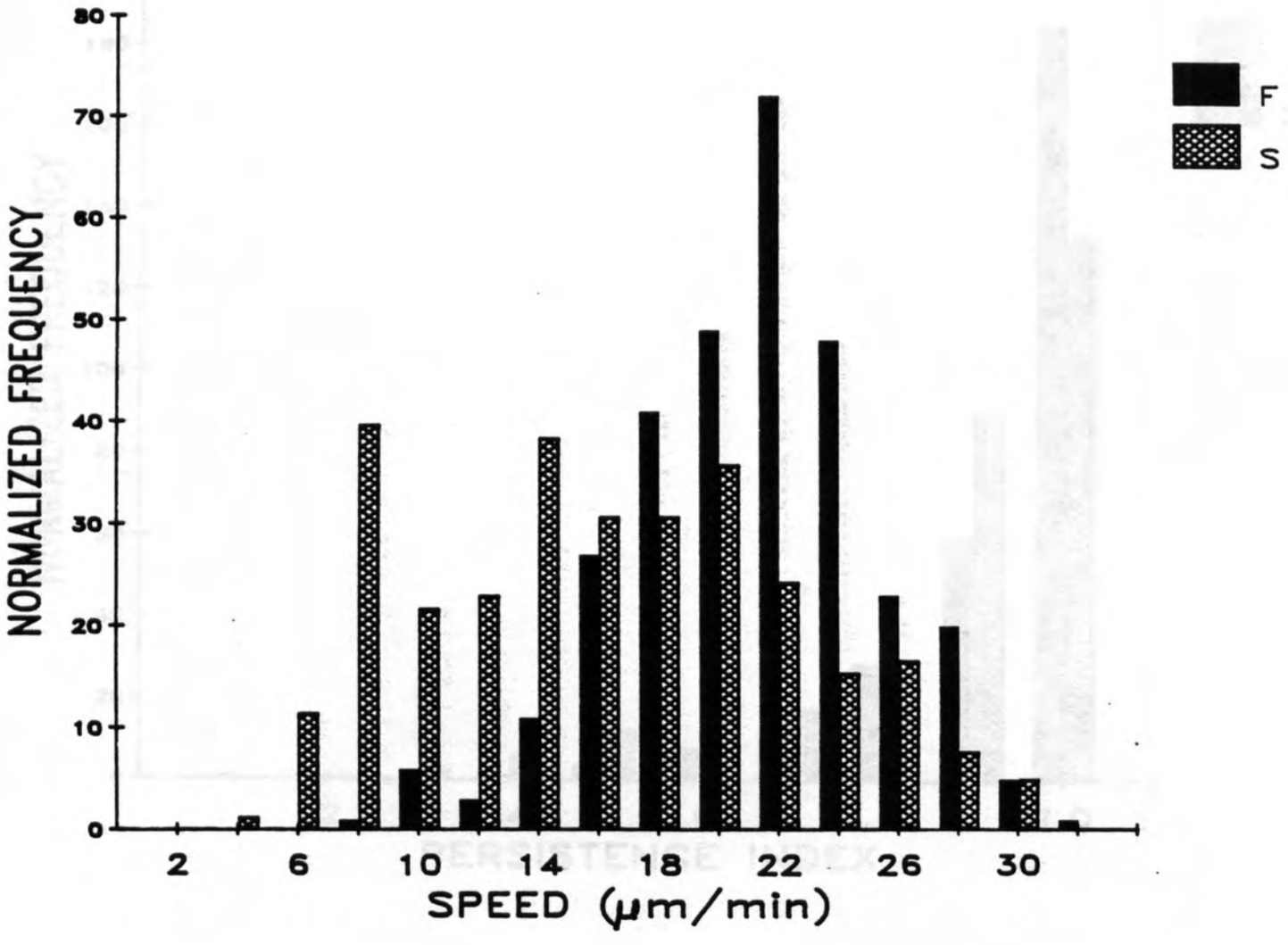

NORMALIZED FREQUENCY
80
70
60
50
40
30
20
10
0
2
6
10
14
18
22
26
30
SPEED (μm/min)
F
S

FIGURE 3

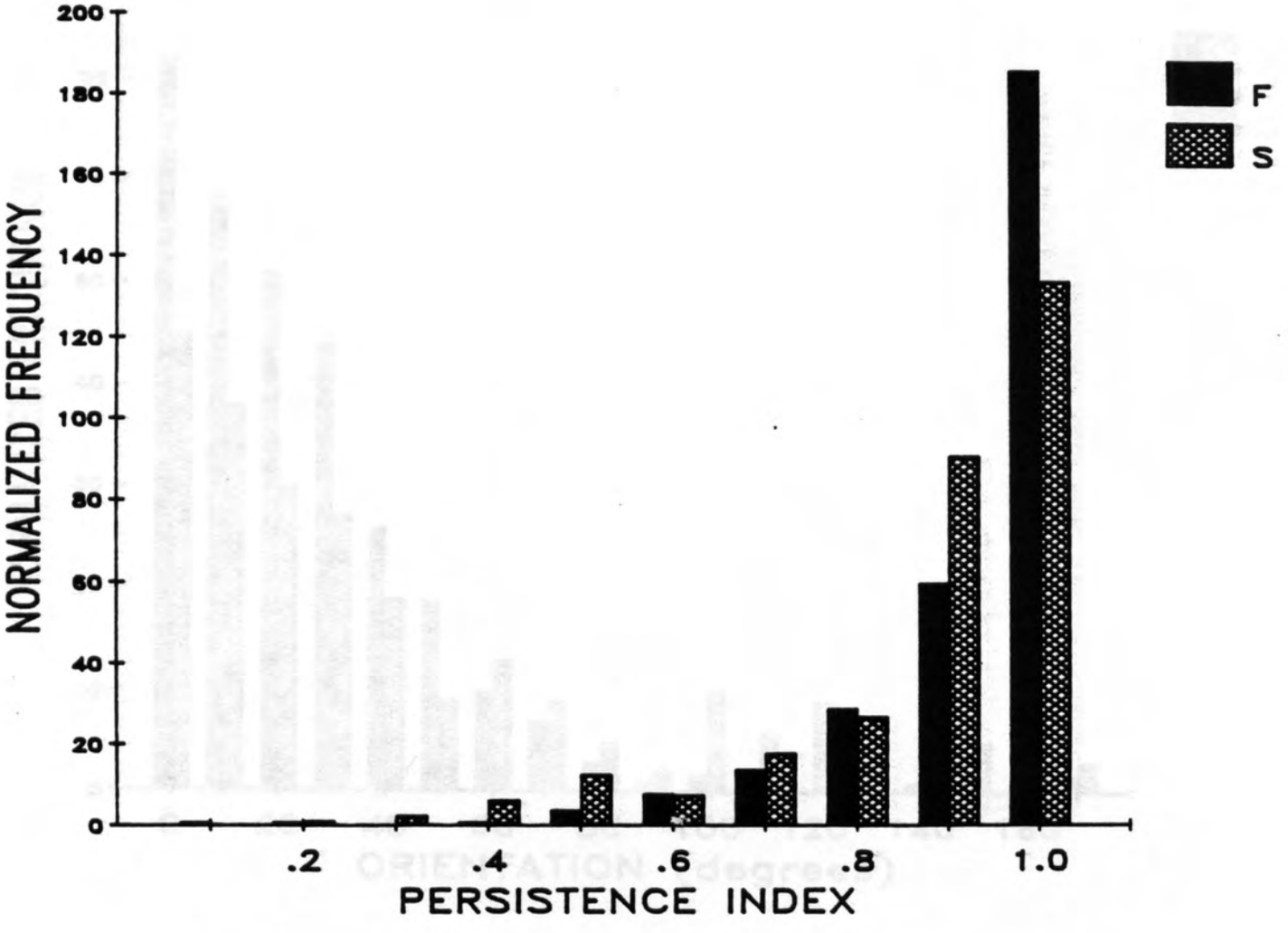

200
180
160
140
120
100
80
60
40
20
0
NORMALIZED FREQUENCY
.2
.4
.6
.8
1.0
PERSISTENCE INDEX
F
S

FIGURE 4

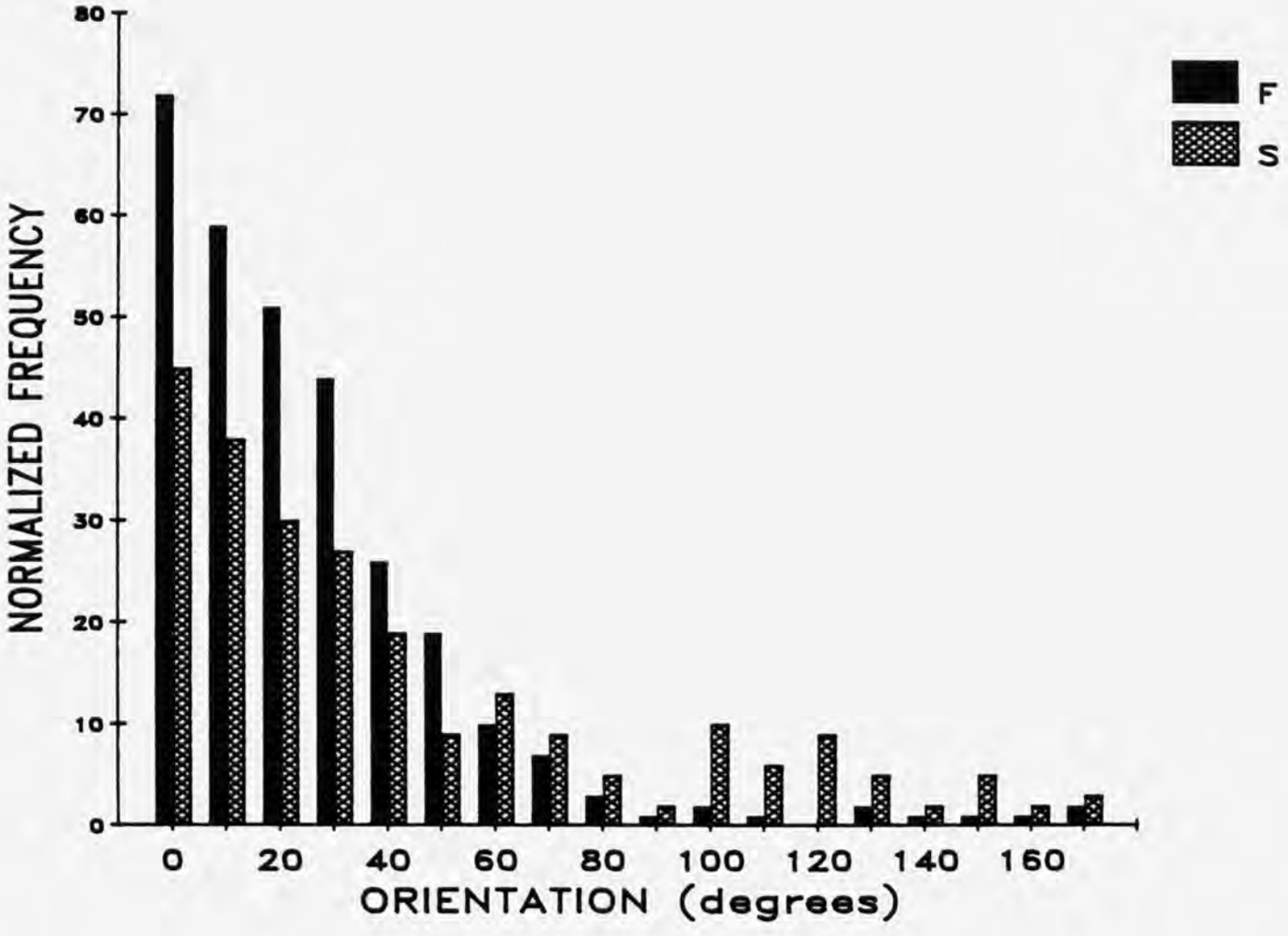
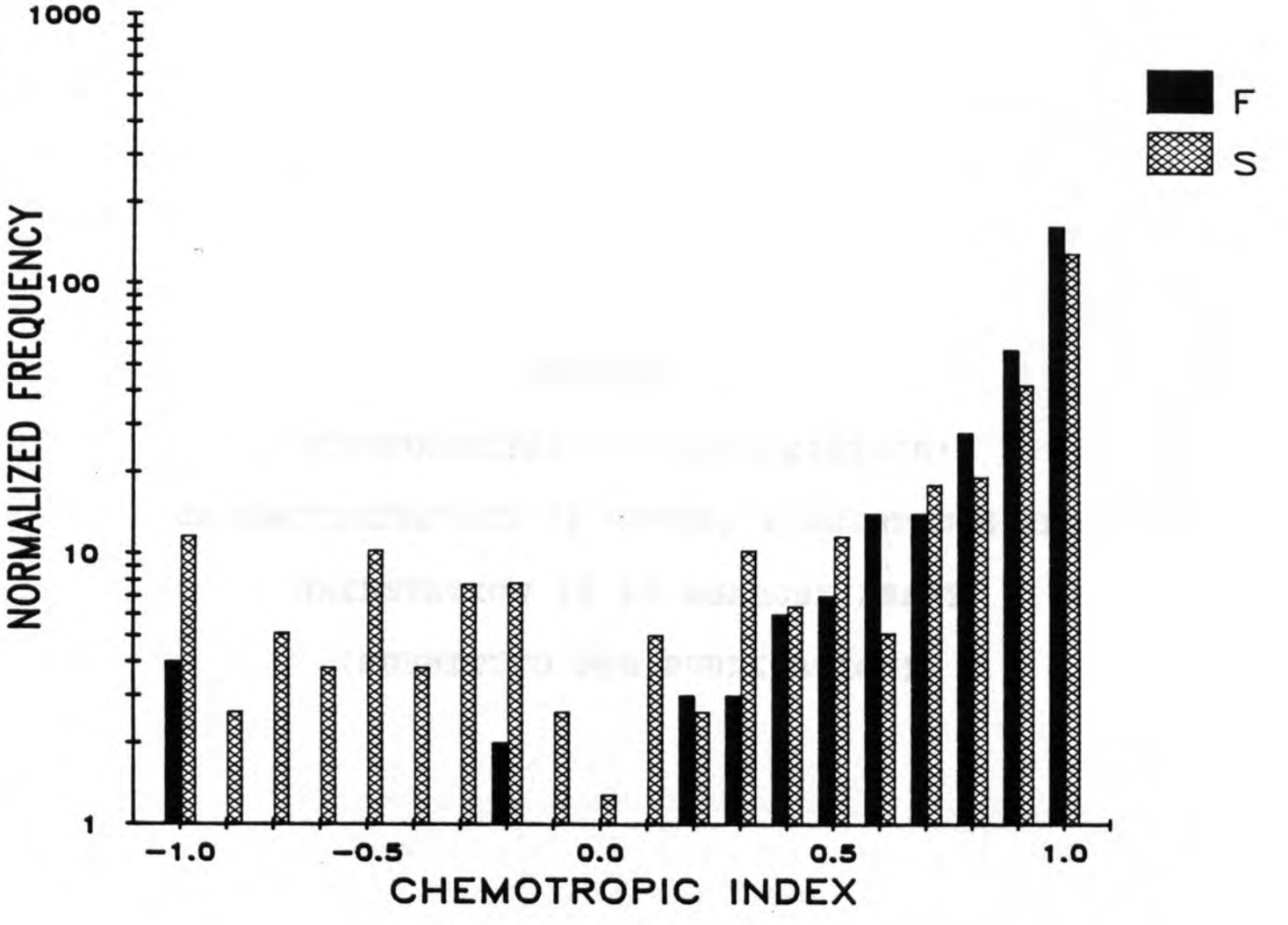

80
70
60
50
40
30
20
10
0
NORMALIZED FREQUENCY
F
S
0
20
40
60
80
100
120
140
160
ORIENTATION (degrees)

FIGURE 5


1000
100
10
1
NORMALIZED FREQUENCY
F
S
−1.0
−0.5
0.0
0.5
1.0
CHEMOTROPIC INDEX

CHAPTER V.

VIDEOANALYSIS OF CHEMOKINESIS: CHARACTERIZATION OF SPEED, PERSISTENCE AND ORIENTATION IN AN AGAROSE ASSAY

(SUBMITTED FOR PUBLICATION)

ABSTRACT

Time-lapse video recording and off-line computer analysis were used to characterise the chemokinetic behavior of individual human neutrophils migrating in an agarose assay. When neutrophils were stimulated with an isotropic concentration of N-fMLP, they migrated at a speed of ~9 um/min and showed no preferred orientation. The ratio of net displacement to total distance traveled was 0.66. Therefore, neutrophils maintained some directional persistence, despite the lack of a gradient of ligand. The speed and persistence of locomotion index were positively correlated, whereas, persistence and orientation were independent. Chemokinesis and chemotaxis can be described as distinct sets of responses which characterise two intrinsic motile states and neutrophils can switch between these two sets of responses.

INTRODUCTION

Kinesis is defined as locomotion which a) lacks definite orientation and b) is dependent upon the intensity of the stimulus. Chemokinesis is a response to an isotropically distributed chemical stimulus in the environment. Chemotaxis is dose-dependent oriented locomotion in response to a gradient of a chemical. Chemotaxis in human neutrophils is the subject of intense investigation due to the importance of this response in inflammation (see Wilkinson, 1982, Keller & Till, 1983, for reviews).

Chemokinesis of neutrophils involves the modulation of the cells speed and turning behavior causing the cells to migrate faster and show an increased tendency for directional persistence, as a function of the ambient concentration of a ligand (Keller & Zimmermann, 1985, Gruler & Bultmann, 1983, Shields & Haston, 1985).

The relationship between chemokinesis and chemotaxis is not fully understood but, if persistent migration is an inherent property of neutrophils, directed migration (orientation) during chemotaxis amy be an inherent property also. Although both chemokinesis and chemotaxis can be initiated by the same chemical, chemokinesis is not simply chemotaxis without a net orientation. Neutrophils may use the information in a chemoattractant gradient to switch between different organizational patterns of the same integrated cytoskeletal /biochemical system. Such states

could distinguish two intrinsic motile states characterised by specific combinations of speed, persistence and net orientation. To determine if two intrinsic motile states exist, we have understaken a rigorous quantitative study of the neutrophils locomotor response to N-fMLP in the presence (Burton, et.al., 1986a) of a gradient.

The purpose of this study is to directly measure the locomotor parameters which characterise the chemokinetic response of neutrophils responding to N-fMLP in the agarose system.

MATERIALS & METHODS

Collection of blood

Venous blood was collected from normal volunteers in accordance with the guidelines approved by the MUSC committee for the Protection of Human Subjects. The neutrophils were isolated as previously described (Burton, et.al., 1986b). Briefly, venous blood was collected in CPD anticoagulant. Neutrophils were isolated by Dextran sedimentation of the red cells followed by density centrifugation of the leukocyte-rich plasma through a discontinuous gradient of Percoll. Washed suspensions of cells consisted of 99% neutrophils, and 98% of the neutrophils were viable immediately after isolation, as determined by the use of an acridine orange/propidium iodide vital staining procedure (Moore, et.al., 1985). Washed neutrophils were suspended in Hanks Balanced Salt Solution (HBSS, Gibco, Grand Island, NY) at 10^7 per ml and assayed within four hrs.

Assay of chemokinesis

Chemokinesis was assayed in an under-agarose system as previously described (Burton, et.al., 1986a). Briefly, a layer of agarose containing N-fMLP was cast in a petri dish, wells were cut in the gel and 10^5 neutrophils were placed in the wells. Preliminary dose-response experiments showed that 10nM N-fMLP produced the maximal response. The dish was incubated at 37°C for one hour, then sealed with Parafilm and arranged on the 37°C heated stage of an inverted

microscope so that the video field was just outside of a randomly selected well, at a random location around the perimeter of the well.

Video Analysis of Locomotion

Time-lapse video recordings were made using an analog enhanced videomicroscope system (Burton & Bank, 1986b) which produced a sharp image of the cells despite the presence of the agarose layer. Plastic sheets were placed over the screen of the video monitor and, during playback of the video recordings, the centers of randomly selected cells were marked at 15 sec intervals for a period of 5 min. The cell paths were retraced, at 1 min intervals, onto the digitizing tablet of a Zeiss Videoplan Image Analysis Computer.

Three fundamental locomotor parameters were determined: Speed, defined as the total distance traveled per min., Velocity, the net displacement per min. and Orientation Angle, the angular component of the Velocity vector. A line from the center of the cell containing well through the center of the video field was defined as 0^{o}, with angles to the left and right defined as negative or positive, respectively.

Two other parameters were derived from the first three. The Persistence of Locomotion (Persistence Index, PI) is the ratio of velocity to speed, and is an indicator of turning. The Persistence Index varies between 1.0 indicating

straight-line motion and 0.0 indicating no net displacement. The cosine of the orientation angle was calculated and is referred to as the Chemotropic index (CI).

The analyses of chemokinesis were based on 3 measurements from 3 donors, with 30-40 cells tracked for each measurement.

Statistical Analysis

The data are reported as means ± the standard deviation. Numerical and graphic analysis were carried out using programs implemented on a Zeiss Videoplan Image Analysis Computer (Sachs, 1982). Deviations from a predicted mean were tested by a single-sided t-test. Goodness-of-fit of data to the line for an equation was determined by a chi-squared goodness-of-fit. Correlations were tested using Pearsons Product-Moment correlation (Pearson's r). Tests of significance or correlations were considered significant at the 95% confidence level.

RESULTS

CHEMOKINESIS

Speed

The mean speed of neutrophils during chemokinesis was 9.6 ± 3.9 um/min (fig. 1). The frequency distribution of speed was a normal curve (Chi-squared goodness-of-fit, df = 11).

Persistence Index

If the cells were turning completely at random, the distribution of PI values would be juniform and a mean of ~0.5 for PI would be observed. The mean persistence of neutrophils during chemokinesis was 0.66 ± 0.25 (fig. 2), which is significantly different from 0.5 (single-sided t-test, $p < 0.05$).The frequency distribution (fig.2) indicated that turning behavior was not random but rather was biased toward higher values. The neutrophils tended to maintain their direction of migration in the absence of a gradient.

Orientation Angle

The mean orientation of neutrophils during chemokinesis was $16.48^{o} \pm 105.7^{o}$. The very large standard deviation indicates a high degree of dispersion. Plot of the distribution of the Orientation Angle (fig. 3) appeared almost flat. The randomness of the distribution was tested by sorting the data into groups at ten degree increments, then calculating an equation for a line with a slope of zero and

a y-intercept equal to the number of cells which would be present in each group if the sample was uniformly distributed across the -180° to 180° range. The deviation of the data from this line was tested using the chi-squared goodness-of-fit. The data not deviate significantly from a line expected for a uniform distribution of orientation values (chi-squared = 16.47, df = 15, $p > 0.05$).

Chemotropic Index (CI)

If the orientation were random, then the mean CI (cosine of the orientation angle) should be zero regardless of the mean orientation angle. The mean CI of neutrophils undergoing chemokinesis was 0.02 ± 0.74, which was not significantly different from zero (single-sided t-test, $p > 0.05$) i.e.: these cells oriented at random. The distribution of the Chemotropic Index is very broad, with small peaks at the extremes (fig 4). These peaks are due to the non-linearity of the cosine function, the slope of which decreases rapidly as the angle approaches zero.

CORRELATION ANALYSIS

Two-parameter scatter-diagrams and correlation analysis were used to determine if relationships exist between parameters. The scatter-diagrams were constructed by assigning X addresses to one variable and Y addresses to the other. Each set of values was then plotted in a two-dimensional array.

Speed vs. Persistence Index

Since, PI is mathematically related to speed (PI = velocity/speed), a correlation would be expected. A weak but significant correlation was found ($r = 0.29$, $df = 113$, $p < 0.05$)(Fig 5). The tendency for faster cells to make fewer/narrower turns can be see in the lack of data points in the lower right quadrant of Fig 5.

Orientation Angle vs. other parameters

Fig 6 shows that there is no relationship between speed and orientation angle ($r = 0.015$, $df = 113$). In the absence of a gradient, the cells orient at random regardless of their speed.

Figs 7 & 8 show that there is no relationship between PI and orientation ($r = 0.007$, $df = 113$) or PI and CI (r 0.08, $df = 113$) respectively. Even those cells with the higher PI values (> 0.8) showed no particvular preference of orientation angle.

DISCUSSION

CHEMOKINESIS IN THE AGAROSE SYSTEM

The mean chemokinetic speed of neutrophils migrating under agarose was 9.6 um/min. Their actual path was ~65% efficient in producing net displacement and the cells showed no preferred orientation (CI = 0.02) during the 5 min observation period.

Why study chemokinesis?

Although a number of investigators have measured the locomotor behavior of neutrophils undergoing chemokinesis, no comparable measurements have been published for the chemokinesis of neutrophils under agarose. Such measurements are necessary if the relationship, if any, between chemotaxis and chemokinesis is to be determined. We have reported previously (Burton, et.al., 1986a) the chemotactic speed, PI and CI of neutrophils in the agarose system. The chemotactic speed (~20 um/min), PI (0.88) and CI (0.79) were much higher than the values reported here for chemokinesis.

We suggest that the relationship between chemokinesis and chemotaxis could be either 1) a gradual increase in speed and persistence resulting from increased orientation or 2) the cells switch between two different kinds of response. The characterization of chemokinesis would provide insights into the motile behavior of the cells in the absence of a gradient.

Several systems have been described for estimating the

locomotor parameters of populations of cells by fitting the spatial distribution of a population of cells to an equation which models the cells motion as a biased random-walk or a flux (Scheiner, Kalager & Vaula, 1980, Lauffenburger, 1983). These methods are limited by the assumption that the behavior of individual cells in a population is homogeneous. Direct measurements of the behavior of individual cells avoid this limitation.

Speed

Previous studies of chemokinesis have reported a wide range of speeds in response to a variety of chemoattractants (cf. Allan & Wilkinson, 1977, casein, 10.8-15.6 um/min; cf. Gruler, et.al., 1984, 10nM N-fMLP, 30 um/min; cf. Howard, 1986, N-fMLP, 9.5 um/min, cf. Keller, et.al., 1984, 10 nM N-fMLP, 23.7 $\pm$ 1.8 um/min; cf. Shields & Haston, 1985, 10 nM N-fMLP, 6.36 um/ 40sec). These differences may be due to differences in experimental conditions used by the different investigators.

For example, Howard (1986) used a Dvorak-Stotler chamber and a flow-through perfusion system to rapidly alter the N-fMLP concentration and measure the acute effects of stimulation. The agarose system is a steady-state assay which does not reveal such acute or transient effects.

Persistence

If the extent of turning was random, one would expect PI values to be evenly distributed throughout the 0.0 to 1.0

range. This would produce a mean PI of ~0.5. However, we calculated a mean PI of 0.66. These results are consistent with a recent report that neutrophils show a reponse called klinokinesis, a change in the number and size of turns which the moving cell makes in response to a stimulus (Keller, et.al., 1984). This response also has a dose-response relationship such that sub or supraoptimal doses of chemoattractant (specifically N-fMLP) stimulate migrating neutrophils to make more/wider turns than does the optimal dose (Shields & Haston, 1985).

This tendency to show some directional persistence in the absence of a gradient may be due to the inherent polarity which migrating neutrophils display (Zigmond, et.al. 1981). Neutrophil polarity has been shown to be a transient motile state which decays with a characteristic time of ~30 sec (Gruler, et.al., 1984). Thus, persistence can be considered as the time between turns (Dunn, 1983). This tendency for directional persistence may be due to a structural asymmetry within the neutrophil cytoplasm. Parysek and co-workers (1984) reported that neutrophils had vimentin intermediate filaments which were found in a loose "knot" in the posterior portion of "randomly migrating" cells. This could stabilize the inherent polarity of a migrating cell during chemokinesis.

Orientation & Chemotropic Index

The lack of a preferred orientation would be expected

if the distribution of chemoattractant was isotropic. Some bias for an orientation away from the cell-containing well could be expected as the cells "diffused" out of the well. The frequency distribution of orientation does not show any bias; indeed, the distribution fits the equation for a uniform distribution. If the behavior of the cells at the outer edge of the mass of migrating cells were measured, a bias would be expected. Observations made at the edge of the well, after a incubation period, show no bias, ie, no preferred orientation. If the neutrophil is considered to be an information processing system where a gradient of chemoattractant is the input and oriented locomotion is the output, then chemokinesis is a zero-input condition (Gruler, et al., 1983).

CORRELATIONS BETWEEN PARAMETERS

Speed vs persistence

The correlation between speed and persistence is similar to previous reports. Keller and coworkers (1985) reported a strong negative correlation between speed and the ratio of speed to velocity (= 1/ PI), which they call the "klinolocomotion index". They stimulated neutrophils with N-fMLP in sealed slide-and-coverslip preparations, and found that the the higher the mean speed of a sample of cells, the higher their directional persistence (the lower the klino-locomotion index). They calculated that, in their system,

if cells could be stimulated to migrate with mean speeds > 25um/min, they would make no turns.

The "klinolocomotion index" for our data would be 1.515 (1/ 0.66). The correlation which Keller et. al. (cf. Fig 4, Keller & Zimmermann, 1985) found would predict a mean speed of ~12-15 um/min for our cells. However, we measured a mean speed of 9.6 um/min. Apparently, the differences between the agarose system and the "slide-and-coverslip" affect the speed of migrating cells.

Persistence vs. Orientation

In our agarose system, cells migrate in a manner which has been described as similar to diffusion (Dunn, 1983, Gruler, et.al., 1984), but they do not show a bias for the direction away from the "source"; the cell-containing well. Immediately after being placed in the wells, the cells experience a transient gradient of N-fMLP as the peptide diffuses out of the gel and into the suspending medium. The volume of peptide-containing agarose is 100x the volume of the six cell-containing wells. The peptide rapidly equilibates such that the cells will be in an isotropic concentration of N-fMLP. There is probably a net flux of cells out of the well during the preincubation period, prior to the observation period. However, after the one hour preincubation, no bias in orientation was detected during the 5 min. observation period (fig. 3). If a bias in orientation remained, it probably has a characteristic time

much greater than 5 min. and therefore, would require a longer observation period to detect.

In the absence of a gradient of ligand, the neutrophils' persistence and net orientation are independent. If cells showed different degrees of orientation as a function of their tendency to turn, it is possible that one function could mask the other. The two-parameter scatter-diagrams (figs. 7, 8) shows that neutrophils oriented at random regardless of their persistence. This baseline data is necessary for quantitative studies of neutrophil locomotion since any systematic directional bias would confound the interpretation of data from chemotaxis experiments. We found no such systematic bias, so no correction factor is necessary.

CONCLUSIONS

These studies define the combination of parameters for neutrophils undergoing chemotaxis. We are interested in the relationship between chemokinesis and chemotaxis, and have shown that the loss of chemotaxis by neutrophils which have been stored for 24 hr. at 20-22^{o}C is due to a loss of speed and orientation by a portion of the population of cells (Burton, et.al., 1986a). We have postulated that chemokinesis and chemotaxis are distinct intrinsic motile states and suggest that those stored cells which have lost the ability to process the information in a gradient of chemoattractant

not only lose orientation but also migrate at a speed similar to that of normal chemokinetic speed due to the reduced efficiency of the motile system.

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FIGURE LEGENDS

Fig. 1. Histogram of chemokinetic speed of neutrophils

Fig. 2. Histogram of persistence of locomotion index. Note: the values are biased toward higher values indicating that cells maintained some degree of directional perisistence.

Fig. 3. Historam of orientation angle. The distribution of the data fits a uniform distribution; $f(x) = 0.056\ N_o$, $N_o = 115$

Fig. 4. Histogram of chemotropic Index.

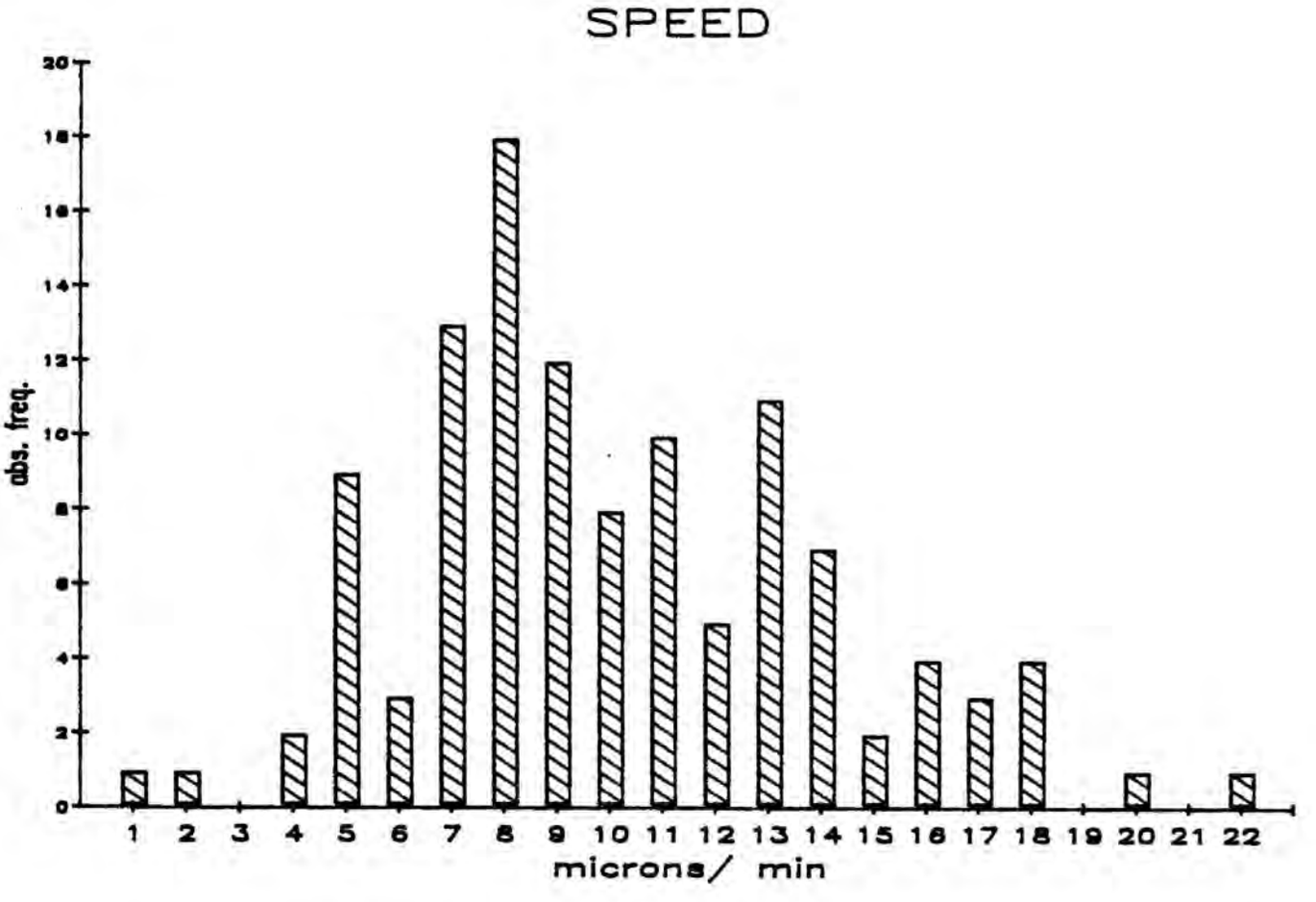
Fig. 5. Scatterdiagram of speed vs persistence index.

Fig. 6. Scatter-diagram of speed vs orientation. Note: Cells orient randomly regardless of their speeds.

Fig. 7. Scatter-diagram of persistence index vs orientation angle. Note: Cells oriented randomly despite the tendency for directional persistence.

Fig. 8. Scatter-diagram of persistence index vs chemotropic index.

FIGURE 1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0
2
4
6
8
10
12
14
16
18
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
microns/ min

FIGURE 2

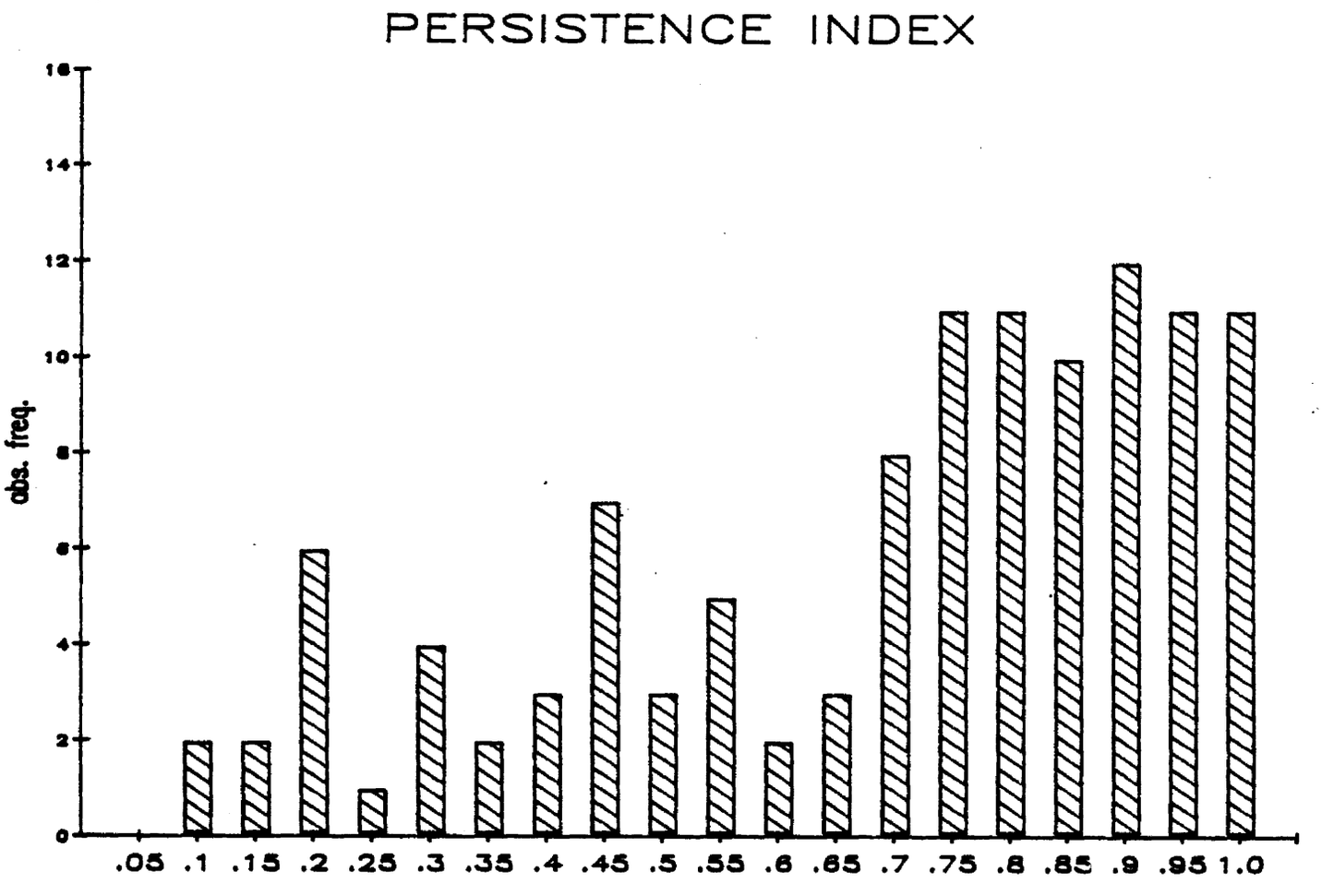
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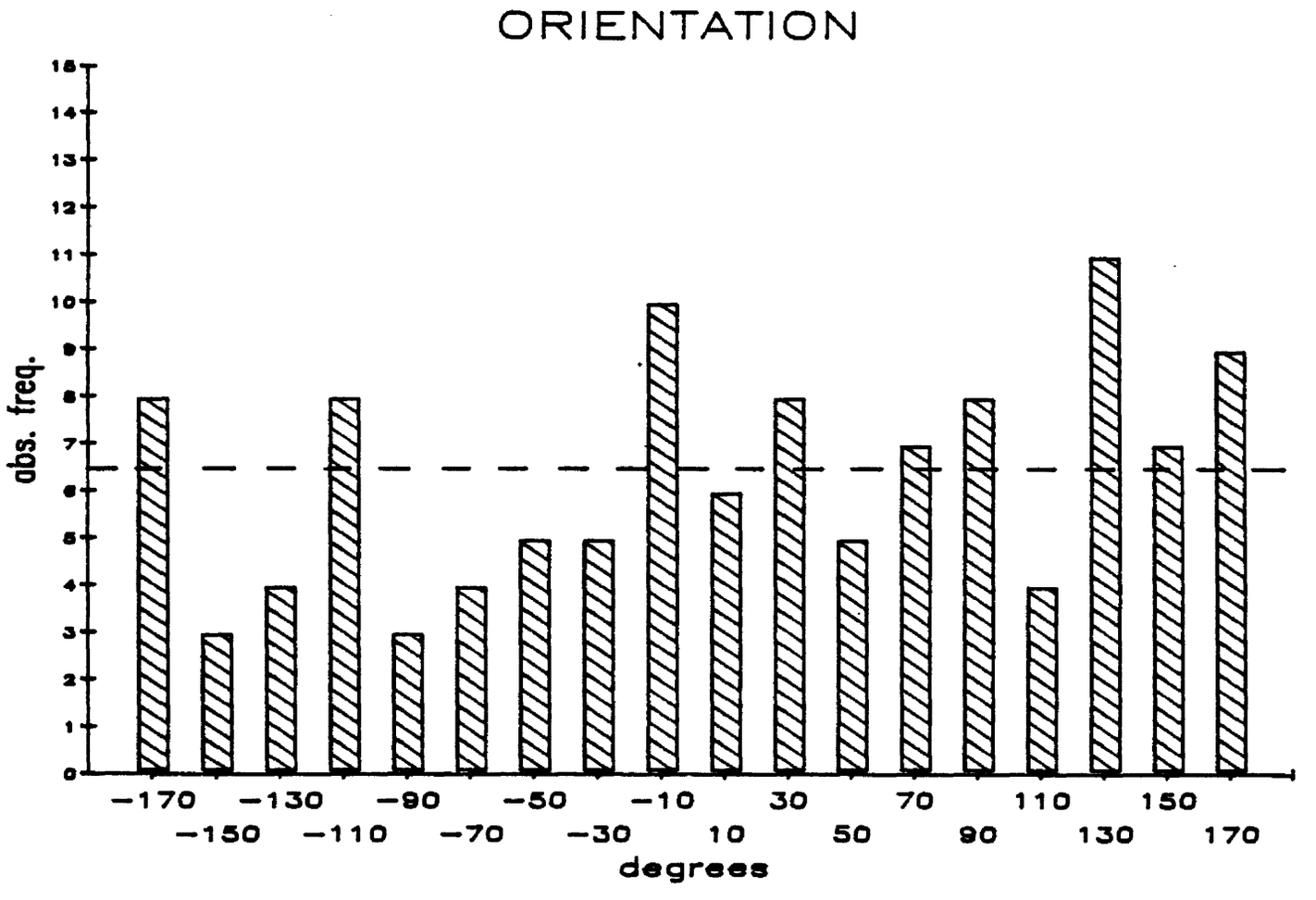
FIGURE 3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150
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degrees

FIGURE 4

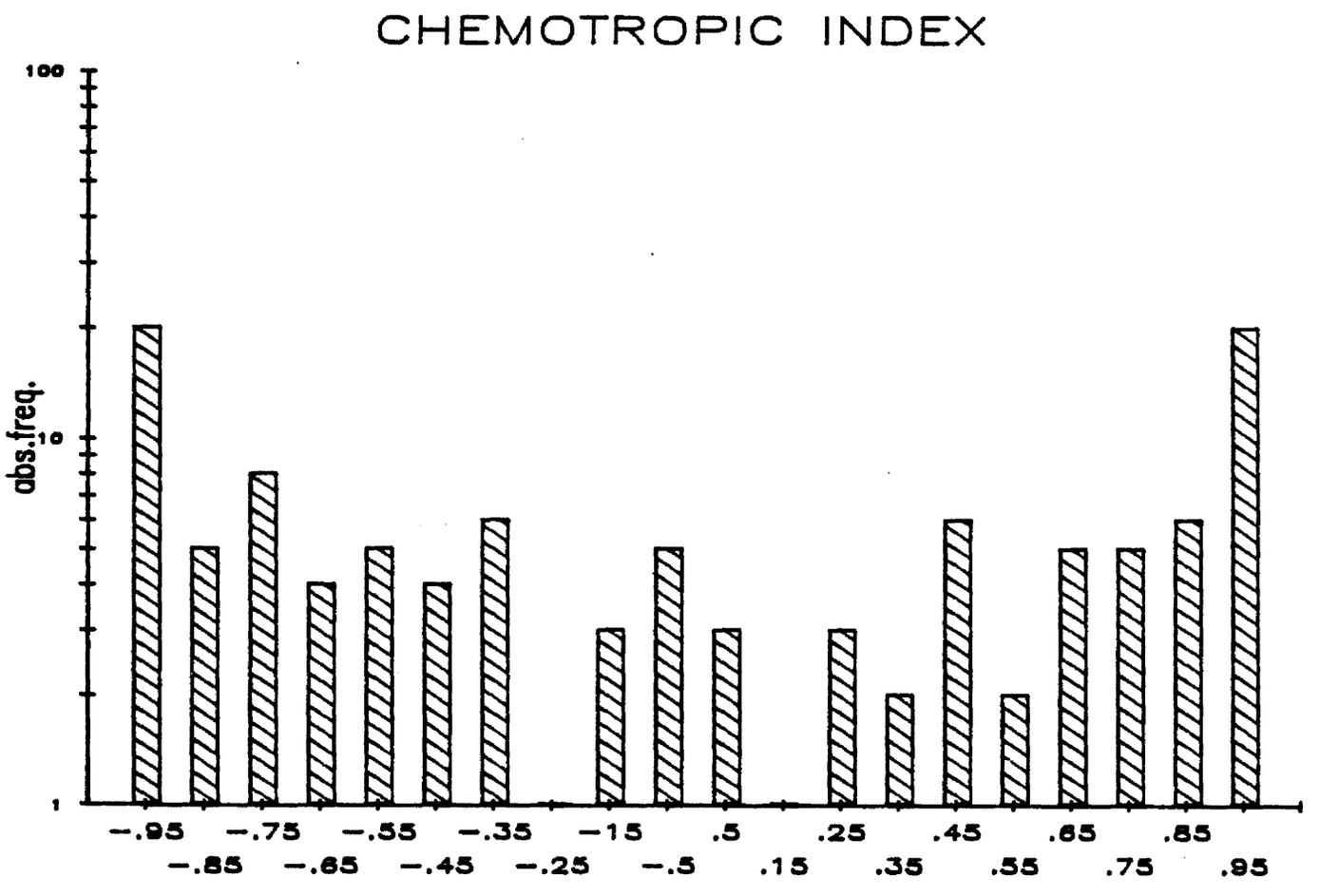
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FIGURE 5

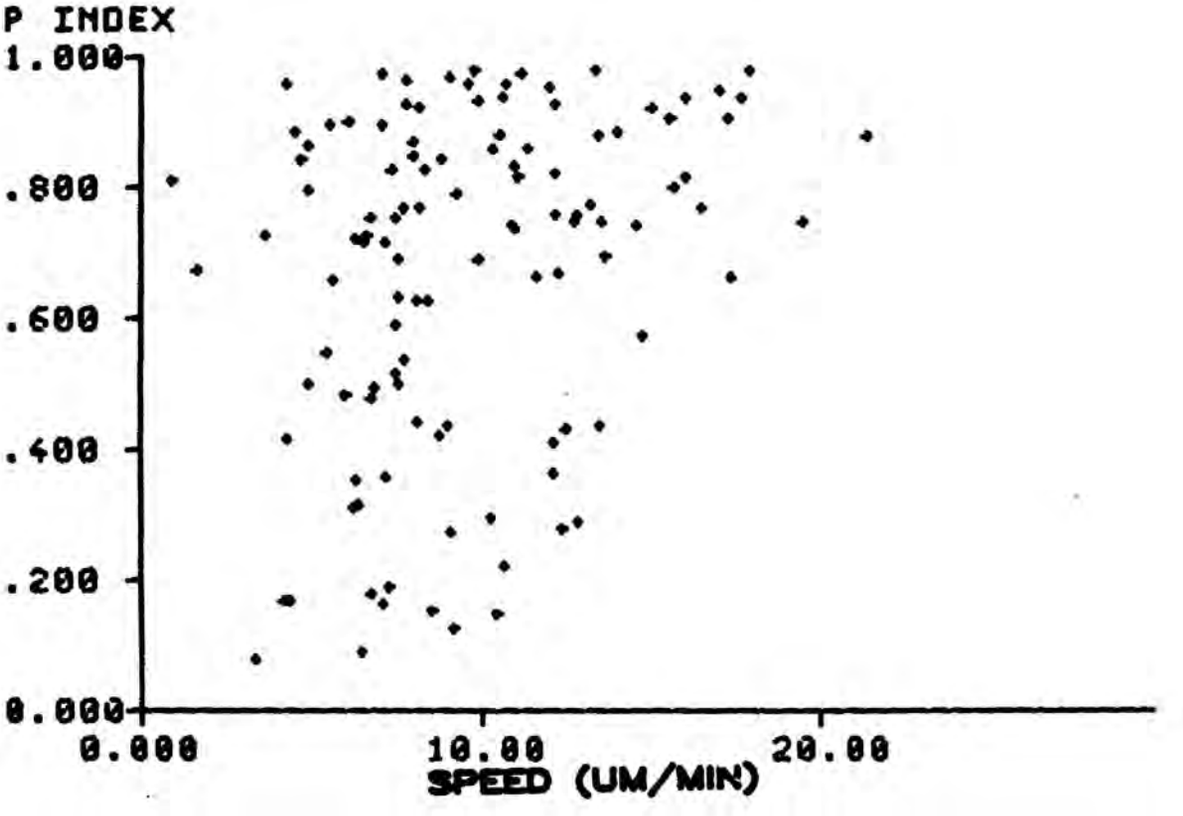
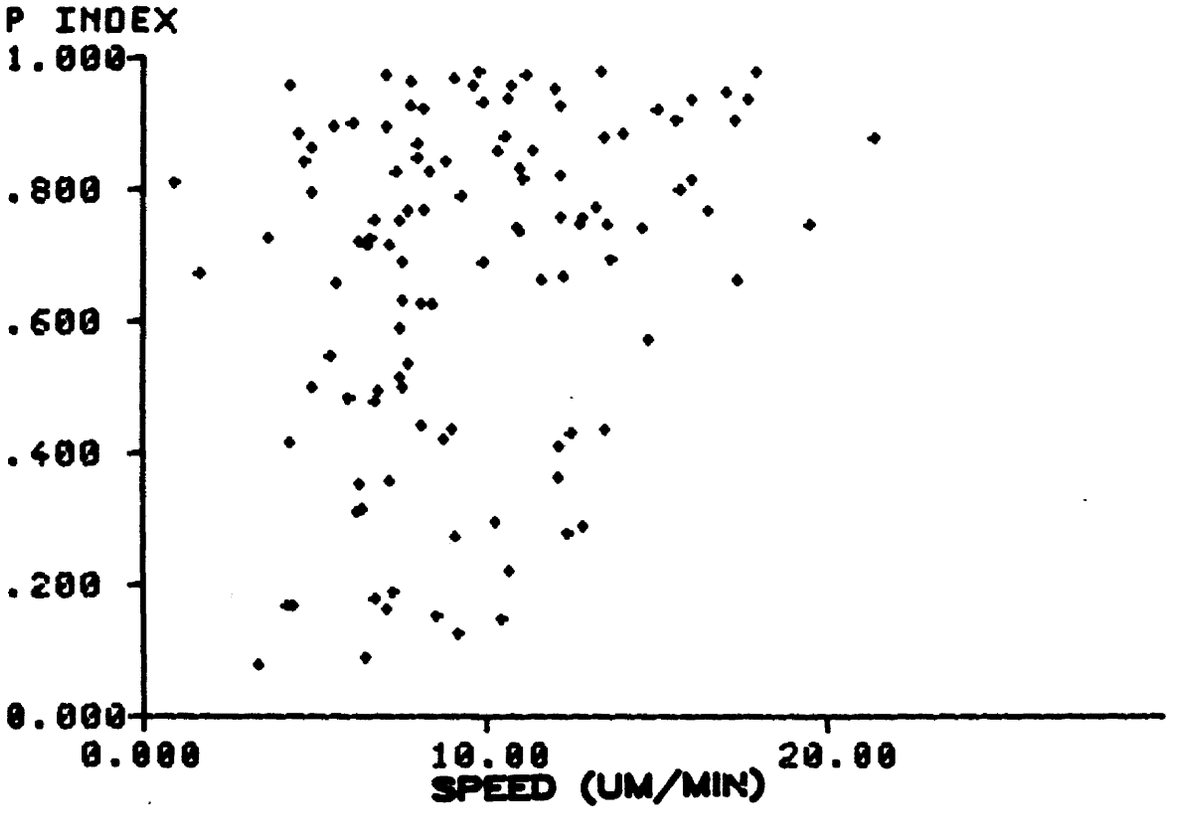


FIGURE 5



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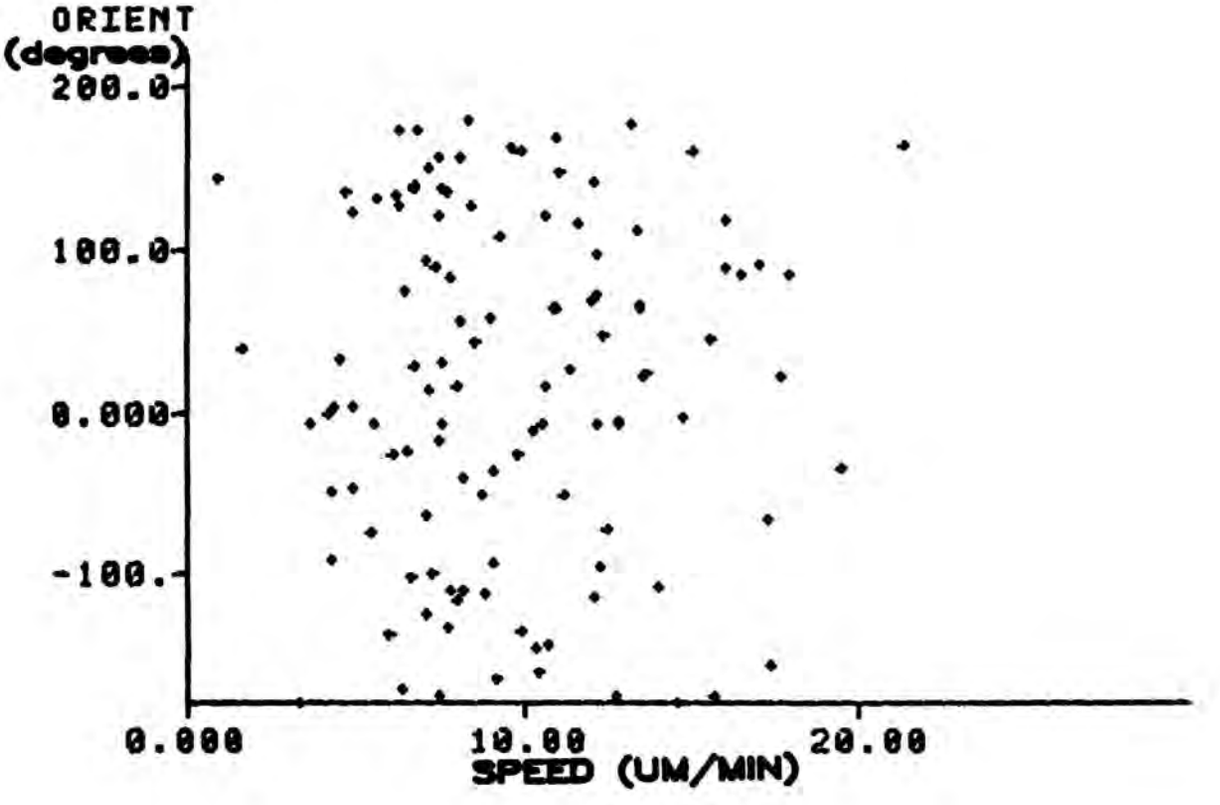
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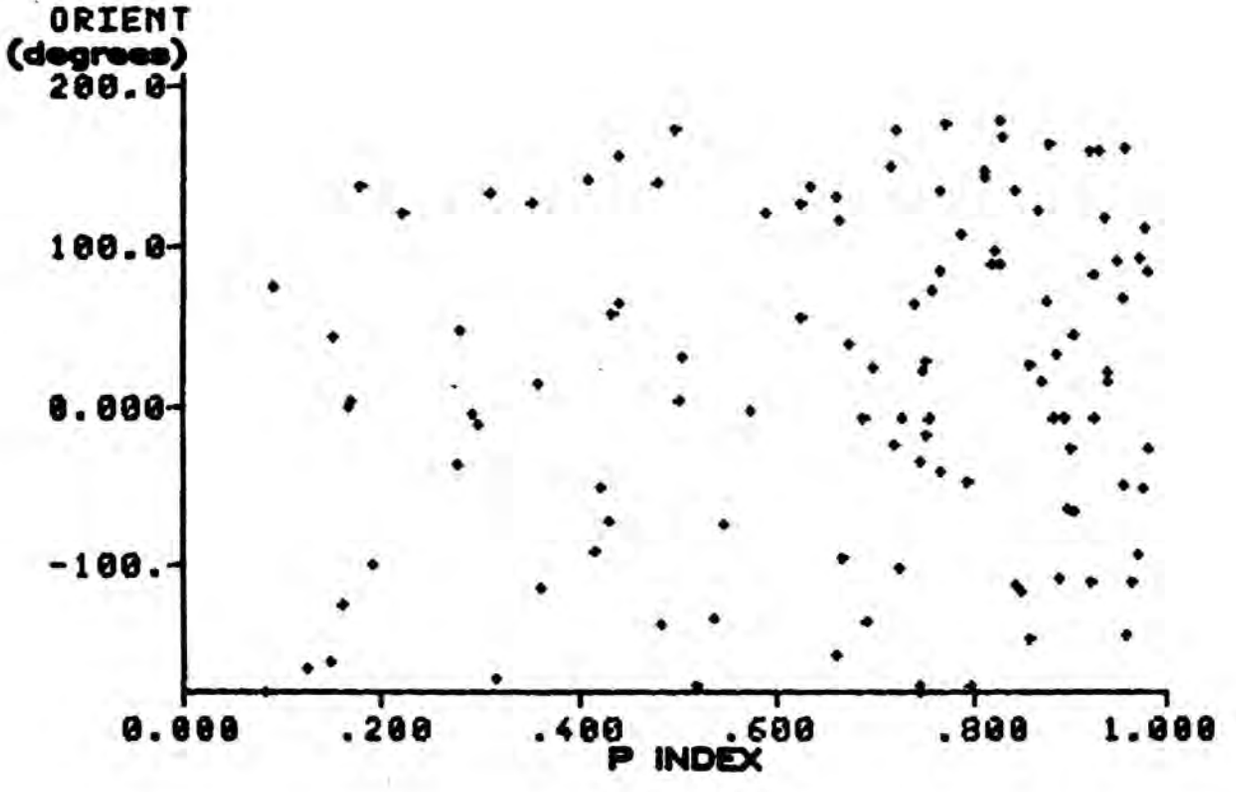
-100.
0.000
10.00
20.00
SPEED (UM/MIN)

FIGURE 7


ORIENT
(degrees)
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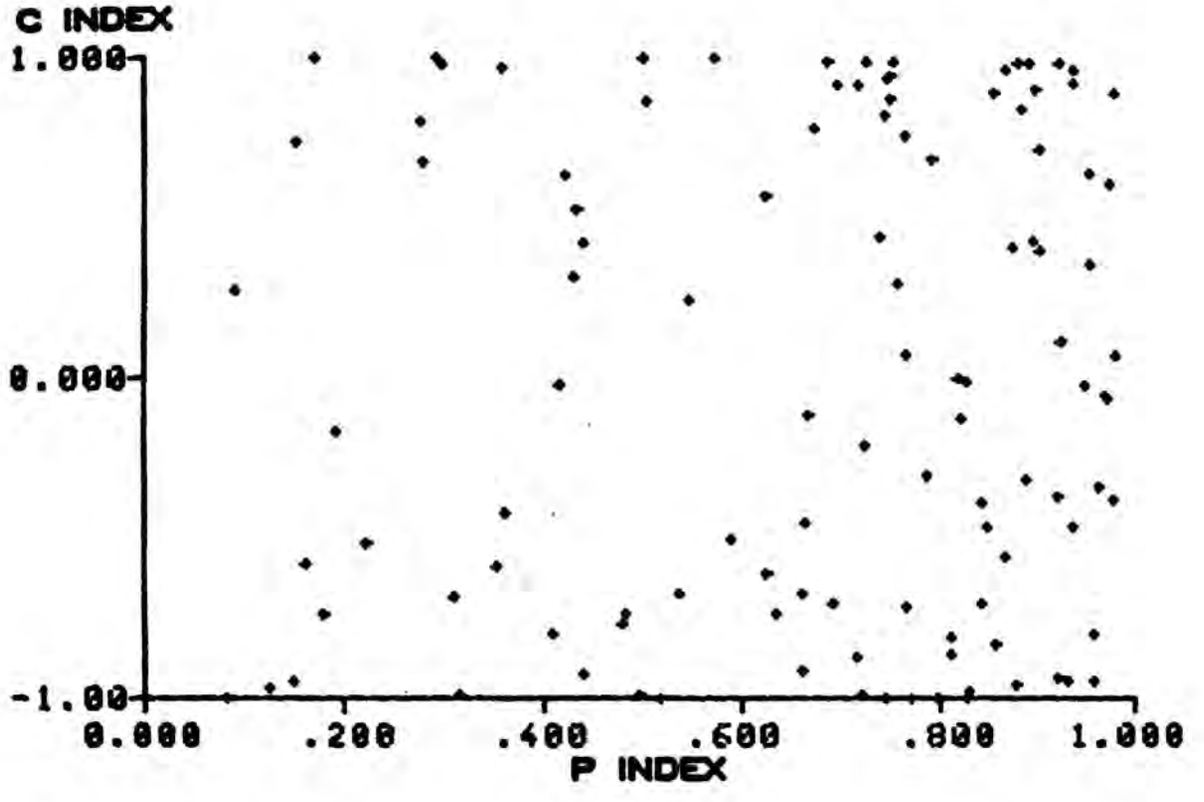
1.000
P INDEX

FIGURE 8



CHAPTER VI.

VIDEO ANALYSIS OF CHEMOTAXIS OF STORED NEUTROPHILS: II. CORRELATIONS BETWEEN PARAMETERS AND THE EXISTENCE OF SUBPOPULATIONS

ABSTRACT

When neutrophils are isolated from the circulation the first function to deteriorate is chemotaxis. In an effort to characterize this loss of function which occurs during storage, we measured three fundamental locomotor parameters: the chemotactic speed, velocity and orientation angle, and calculated the persistence of locomotion index (velocity/speed) and chemotropic index (cosine of the orientation angle) for fresh neutrophils and neutrophils stored for 24 hr. The loss of chemotaxis can be observed by the change in shapes of the frequency distributions of the parameters which characterise the locomotor behavior of stored neutrophils. Our data reveal that:

1. the frequency distribution of speed for individually stored cells had a different shape than tha of fresh cells. A seperate subpopulation of cells which migrated at slower speeds became obvious.

2. the distribution of orientation for fresh cells is normally distributed and contains a small group (~9% the total) of cells which orient at random in a gradient,

3. the chemotactic locomotion of the majority of stored cells is comparable to that of fresh cells, but 35% of the stored cells migrate more slowly and orient at random in a chemoattractant gradient.

4. the persistence and orientation of both fresh and stored cells were not correlated with speed nor can they be predicted from speed and

5. the orientation accuracy (chemotropic index) and persistence are correlated, and this correlation is not altered by storage.

INTRODUCTION

The ability of human neutrophilic granulocytes (neutrophils) to carry out their normal physiological functions deteriorates rapidly during storage regardless of conditions (see Glasser, 1982, for review). The loss of chemotaxis which occurs when neutrophils are stored in plasma at 20-22° C for 24 hr is due, in part, to a decreased number of chemotactically responsive cells (Glasser, 1977). Of those responsive cells, some show a loss of orientation accuracy and they migrate at slower rates (Burton, et.al., 1985a, 1985b, 1986c).

Chemotaxis in human neutrophils involves a series of responses which converts the information in a concentration gradient of a chemoattractant into directed displacement of the cell. Many of the specific components in chemotaxis, ranging from the initial ligand-receptor interaction at the cell surface to the adherence of neutrophils to substrates, have been extensively studied (see Wilkinson, 1983, for review). Nevertheless, the loss of chemotaxis after storage remains an unexplained phenomenon despite attempts to identify the cause (Glasser, et.al., 1983).

To characterize the effect of storage, the changes several locomotor parameters of neutrophils undergoing chemotaxis were measured after storage for 24 hr at 20-22° C (Burton, et. al., 1985b, 1986c). The decrease in chemotaxis is due to a decrease in speed and a loss of orientation by

some cells. We have suggested that room temperature storage induces a shift of cells between pre-existing subpopulations of neutrophils (Burton, et.al., 1986c). Such subpopulations were characterised by their response to chemotactic stimuli: one group shows fast, accurate locomotion, while the other group shows slower, randomly oriented locomotion comparable to chemokinesis. We noted in a preliminary report (Burton, et.al., 1986c) that a larger database and more rigorous numerical methods were necessaary to detect correlations and subpopulations.

In this report we verify the existence of these subpopulations, describe how different locomotor parameters are related to each other and show how these relationships and subpopulations are altered by storage at 20-22^{o} C for 24 hrs, based on a larger sample of cells.

MATERIALS AND METHODS

Collection of blood

Venous blood was collected from normal volunteers in accor- dance with the guidelines of the MUSC committee for the Protection of Human Subjects. Neutrophils were isolated as previously described (Burton, et.al., 1986c). Briefly, venous blood was collected in CPD anticoagulant. Neutrophils were isolated by Dextran sedimentation of the red cells followed by density centrifugation through a discontinuous gradient of Percoll. Washed suspensions of cells consisted of 99% neutrophils, and 98% of those neutrophils were viable immediately after isolation, as determined by acridine orange/propidium iodide staining (Moore, et.al., 1985). Washed cells were then suspended in Hanks Balanced Salt Solution (HBSS), [Gibco, Grand Island, NY] at 10^7 /ml. The neutrophils were either assayed for chemotactic motility within four hrs, or were stored in ABO-compatible CPD-A1 plasma at 2-5 million cells per ml and stored at 20-22° C. in a polyvinyl chloride blood storage bag (Delmed, T3101, Canton, MA) for 24 hr.

Assay of chemotaxis

Chemotaxis was assayed by an under-agarose system (Nelson, et.al., 1977) as described previously (Burton, et.al., 1986b). Briefly, a layer of agarose was cast in a petri dish, rows of 3 wells were cut in the gel, 10^5 neutrophils were placed in the center well, the chemoattractant (1

uM N-fMLP, 10ul) in the outer well and HBSS in the innermost. The dish was incubated at 37° C for one hour, then sealed with Parafilm and arranged on the heated (37° C) stage of an inverted microscope so that the field of view was just outside the cell containing well, between the cell and chemoattractant containing wells.

Video Analysis of Chemotactic Locomotion

Time-lapse video recordings were made using an analog enhanced videomicroscope system (Burton & Bank, 1986a) as described previously (Burton, et.al., 1986c). Kinematic analysis of the motion of the neutrophils was performed using a Zeiss Videoplan Image Analysis Computer. The statistical analyses are based on 11 pairs of measurements (fresh vs stored) from 8 individual donors, with 40-50 cells tracked for each measurement (Fresh =546 cells, stored = 452 cells). Three fundamental locomotor parameters were measured: Speed, defined as the total distance traveled per min., Velocity, the net displacement per min., Orientation Angle, the angular component of the Velocity vector. The line between the center of the chemoattractant containing well and the cell-containing well was defined as 0°, with angles to the left and right defined as negative or positive, respectively.

Two other parameters were calculated from the speed, velocity and orientation angle. The ratio of velocity to speed was defined as the Persistence of Locomotion Index

(PI). PI is an indicator of turning behavior which varies between 1.0, indicating straight-line motion, and 0.0, indicating no net displace- ment. The cosine of the orientation angle was defined as the Chemotropic index.

Statistical analysis

Statistical and graphic analysis of data was carried out on the Zeiss Videoplan Image Analysis Computer (see Sachs, 1982, for specific procedures). The two and three parameters plots were generated using the Videoplan interfaced to an Okidata 82 printer. The frequency distributions were generated with Chart-Master (Decision Resources, Westport, CT) running on an IBM-PC microcomputer interfaced to a Hewlett-Packard model 7475A plotter.

Frequency distributions were tested for normality or fitted to theoretical equations by means of the Chi-squared goodness-of-fit test for a Gaussian hypothesis. The one-sample Komolgoroff-Smirnoff goodness-of-fit test was used to determine if a portion of a given frequency distribution fit a Gaussian curve. Differences between parameters for fresh and stored cells were tested with non-parametric procedures: 1) the Wilcoxin-Mann- Whitney procedure (U-test) was used to test the significance of differences between medians of non-Gaussian distributions with the same shape, 2) the two-sample Komolgoroff-Smirnoff procedure (K-S test) was used to determine if two samples were equally distributed and drawn from the same population. Correlations

between means of speed, persistence of locomotion and chemotropic index were tested using Sperman's rank-order correlation. Non-parametric test were employed because some of the distributions were non-Gaussian. Differences and correlations were considered significant if $p < 0.05$.

RESULTS

INDIVIDUAL PARAMETERS

Speed

The mean speed of the fresh cells was 21.7 ± 6.2 um/min (range = 6.5 to 39 um/min). The speed of fresh cells was normally distributed (Chi-squared test, df= 18). The mean speed of stored cells was 14.56 ± 5.66 um/min, (range = 0 to 30 um/min) (Fig 1). Stored cells showed a broad, non-Gaussian (df = 7) distribution (Fig 1). The median speed of fresh cells (21.39 um/min) was significantly different (U-test) from that of stored cells (14.75 um/min).

Persistence of Locomotion (PI)

This parameter is an indicator of turning behavior; ie, high persistence indicats that few/narrow turns were made. The mean PI of fresh cells was 0.87 ± 0.13 with a narrow, skewed distribution (Fig. 2). The mean PI for stored cells was 0.80 ± 0.2. Significantly more stored cells had PI values below the common median (PI = 0.90) than fresh cells. The fresh and stored cells were neither equally distributed nor drawn from the same population (two-sample K-S test).

Orientation

The mean orientation angle (Fig 3) of fresh cells was $8{\cdot}1^{o} \pm 43.0^{o}$. The orientation was symmetrical around 0^{o} with positive and negative orientations occuring with equal probability. The distribution of the orientation angles is

a non-Gaussian curve (Chi-squared test, df = 18).

The mean orientation of the stored cells was -4.1° ± 66.6°. The distribution for stored cells is non-Gaussian (Chi-squared test, df = 13). When compared to fresh cells, a higher percentage of stored cells oriented at angles > 90° (fig. 3). Although the difference between medians was significant (Fresh = 6.6° vs. Stored = -2.1°, U-test, $p > 0.05$), tests for differences in central tendency are not the most sensitive indicator of differences in orientation accuracy, since this parameter is symmetrical around 0°.

Chemotropic Index (CI)

The proportion of stored cells which oriented away from the chemoattractant can be better characterized when the data is transformed to emphasize the dispersion of the distribution. We used a cosine transform of the orientation angle of each individual cell, the chemotropic index (CI), (Burton, et.al., 1986c) to assess this dispersion more accurately. The product of CI x PI is equivalent to the "Chemotropism Index" defined by Nossal and Zigmond (Nossal & Zigmond, 1976) or the "Degree of Orientation" defined by Bultmann (Bultmann, 1983).

The mean CI for fresh cells was 0.79 ± 0.39 vs 0.55 ± 0.59 for stored cells. The frequency distributions (Fig. 4) show that more stored cells migrated away (CI < 0) from the chemoattractant. Significantly more stored cells had CI values less than the common median (CI = 0.89) than fresh

cells, therefore, the two samples were neither equally distributed nor drawn from the same population (two-sample K-S test).

EXISTENCE OF SUB-POPULATIONS

Speed

The distribution of speed of stored cells appeared to be bimodal (Fig. 1). The cumulative frequency plot of the speed of stored cells (Fig. 5) shows some deviation from a single Gaussian curve in the region between 3 to 12 um/min. When a seperate mean and standard deviation was calculated for this range, which contained 35% of the stored cells, the cumulative frequency distribution conformed to a Gaussian curve (fig 5b, N = 147 cells, Mean = 8.24 um/min, SD = 2.09 um/min, one-way K-S test, 99% confidence limit). This confirms that stored cells exist as two subpopulations with different chemotactic speeds.

Orientation

The frequency distributions for orientation of fresh and stored neutrophils are shown in Figs 6a & 6b (with the actual frequencies plotted with broken lines and the Gaussian distribution plotted with dotted lines). Both frequency distributions are non-Gaussian and the distribution for stored cells is offset from the baseline i.e., there is no point with a frequency of zero. If the calculated standard deviations of the orientation of fresh and

stored cells were biased by the presence of cells which migrated away from the chemoattractant (orientation angles > ± 90°) the unbiased dispersion of the data might be less than the calculated standard deviation would suggest. For a given mean, Gaussian equations have higher maxima and become narrower as the standard deviation decreases (Sachs, 1982). If the true dispersion of the orientation of stored cells is less than the calculated standard deviation, then the distribution may, in fact, be normal.

This possibility was tested by fitting Gaussian curves to the observed distributions and adjusting the size of the standard deviation and size of the fraction of randomly oriented cells until the value of chi-squared (a measure of the goodness-of-fit) was minimum (Sachs, 1982). Figs 7a & b (dotted lines) show the curves for the equations which gave the lowest chi-squared values and the best fit. The fraction of cells which oriented randomly was greater for the stored neutrophils (35% vs 9%).

The standard deviations for the best fit to a Gaussian curve were similar for fresh (30°) and stored (32°) cells. This indicates that the precision orientation of the majority of stored cells is quantitatively similar to that of fresh cells, and therefore, their ability to orient was not compromised by storage.

CORRELATION ANALYSIS

Two-parameter scatter-diagrams were used to determine if a relationship exists between different pairs of parameters.

Speed vs. Persistence of Locomotion

Since PI is mathematically related to speed (PI = velocity/speed), a significant correlation was expected, but not found, (Fresh: $r_s = 0.475$, df = 9, $p > 0.05$ vs stored: $r_s = 0.598$, df = 9, $p > 0.05$). Most of the cells were highly persistent regardless of their speed (figs. 8a & 8b), but more of the slower cells show lower persistence after storage.

Speed vs. Orientation

When speed was plotted against orientation (figs. 9a & 9b), no correlation can be seen for either fresh or stored cells. Most cells oriented toward the source (0^o) regardless of speed. Fig. 9b shows many more stored cells oriented at angles greater than $\pm$ 90^o and those cells tended to move slower.

Speed vs. Chemotropic Index

Speed and chemotropic index are also not linearly correlated for either fresh (fig 10a) or stored (fig 10b) cells (fresh: $r_s = 0.411$, df = 9, $p > 0.05$ vs stored: $r_s = 0.584$, df = 9, $p > 0.05$). Most cells have a high CI, regardless of speed, while the cells with negative CI tend to be slower. Almost all of the cells which did not orient

accurately (CI < 0) had speeds < 20 um/min.

Orientation Angles vs. Persistence

When orientation angles were plotted against PI for fresh cells (fig 11a), the persistent cells tended to have smaller orientation angles. Some of the cells were neither persistent nor did they orient accurately and the number of these cells increased substantially after storage (fig 11b).

Persistence Index vs. Chemotropic Index

PI and CI are measured independently of each other, but are correlated statistically, by rank-order, for both fresh (fig 12a) and stored (fig. 12b) cells (fresh: $r_s = 0.74$, df = 9, $p < 0.05$ vs stored: $r_s = 0.788$, df = 9, $0.05 > p > 0.02$), ie; persistent cells are accurately oriented cells. Most motile cells were persistent, and oriented accurately, while the remainder of the motile cells seem to orient and turn randomly. Thus many more stored cells turned and oriented randomly.

3-D Frequency Histograms

To produce this kind of histogram, the data was sorted by using two different parameters as X and Y addresses in a matrix, as in the the 2-D scatter diagrams. Each cell was allocated to a category defined by a given pair of parameters. The number of cells in each category is displayed on a Z axis. After the cells are categorized, the number of cells and the proportion of a population within a given range of X and Y values can be quantified.

Speed vs Persistence Index

Fig. 13 a and b are the frequency histograms for speed vs persistence index for fresh and stored cells. While 52% of fresh cells had speeds between 14 and 26 um/min and PI > 0.85, only 42% of stored cells had comparable values. This indicates a small decrese in speed even for persistent cells after storage.

Persistence Index vs. Orientation Angle

The frequency histograms (figs 14, a and b) of persistence and orientation shows the clustering of persistence and orientation values, 80% of the fresh cells have persistence indices > 0.80 and orientation angles $\pm$ 45^{o}, while 56% of stored cells had comparable values.

Persistence Index vs. Chemotropic Index

The 3-D histogram of persistence index and chemotropic index (fig 15, a and b) shows that 68% of the fresh cells had PI > 0.85 and CI > 0.80. After storage, only 39% of the cells retained comparable values.

DISCUSSION

There are two basic approaches for assessing the chemotactic response of neutrophils. Population assays measure the average behavior of a group of cells migrating through filters or under agarose. These methods are convenient and are used extensively to estimate how groups of cells behave. The interpretation of such indirect methods is limited by the assumptions which must be made about the cells behavior, ie.; whether an individual cells behavior varies over time or whether cells are hetergeneous in their motile behavior. The single cell assay involves visualization and measurement of the behavior of individual cells. Such direct measurements are not dependent upon a model behavior of the system and are limited only by the reproducibility of the assay conditions and the the number of cells needed to generate a statistically meaningful conclusions.

We made direct measurements of the behavior of individual stored neutrophils in an effort to identify what was the unique attribute of chemotaxis which was sensitive to storage. Our results show that: 1) the orientation accuracy (chemotropic index) and persistence of a given cell <u>cannot</u> be predicted from speed, and are possibly independent of speed, 2) persistent cells tend to orient accurately, 3) room temperature storage induces a change in some cells which is manifest as a decrease in speed, persistence and

orientation accuracy, 4) room temperature storage uncouples orientation accuracy from persistence in some cells, 5) the distribution of the speed of stored cells is bimodal. With 35% of the stored cells forming a distinct, normally distributed group within the larger population, 5) the orientation angle of both fresh and stored cells is normally distributed with approximately the same standard deviation, 6) there is a pre-existing subpopulation of fresh cells (~9%) which orients at random in a chemotactic gradient. After room temperature storage more cells (~35%) are present in that randomly oriented subpopulation.

Relationship of persistence and chemotropic indices to speed

The force required to produce neutrophil locomotion is generated by an actin-myosin system, which operates by extending a lamellipod from the "anterior " cell surface in the region of ligand-receptor interaction, then pulling the posterior region of the cell "forward" by a calcium-dependent contraction (Oster, 1984). This contraction manifests as a wave which propagates from the lamellipod through the polymerized actin-rich cell cortex to the tail of the activated cell. Our observation that turning (persistence) and orientation were independent of the rate of chemotactic locomotion (figs. 7,8,9) implies that the motion producing actin-myosin system is not directly responsible for the turning and direction finding functions of neutrophils.

Relationship of persistence index to chemotropic index

The persistence and chemotropic indices (figs. 11a & 11b) are significantly correlated by rank-order (Sperman's Procedure). Storage alters this relationship such that those cells which are unable to respond to the chemotactic gradient also turn at random. This explains why the rank-order coorelation is maintained after storage, and suggests that the loss of orientation accuracy is the effect of the loss of the integration of the "steering mechanism" and the "intracellular compass". Such a concurrent change in behavior could be accounted for by an alteration of cytoskeleton elements which affects both turning and orientation. The likely candidates are the microtubules and intermediate filament complex:

1)Microtubules

Allan & Wilkinson, (1977) showed that exposure to antimicrotubule agents causes migrating neutrophils to make more turns (lower PI), but such cells were still able to locate the chemoattractant source. Since Anderson (Anderson, et.al., 1982) found that when neutrophils were oriented in an N-fMLP gradient, their microtubules were elongated in the direction that the cell was moving and shortened in the direction perpendicular to the movement. Thus, the microtubules are probably involved in maintaining but not in establishing orientation.

The inherent cellular polarity of activated neutrophils has also received a great deal of attention. Depolymeriz-

ation of the microtubules can activate the actin-myosin "motor" (Haston & Shields, 1985, Keller, et.al., 1984) and cause the cell to assume a polarized configuration. This suggests that the microtubules restrain cellular motion until an activation signal is received. This "polarized configuration" is more stable in a chemotactic gradient, since the waves of contraction originated from the same region in the anterior of the cell (Haston & Shields, 1985). A structural asymetry could maintain this stable "polarized" form, limit turns and increase the efficiency of the actin-myosin "motor".

The role of microtubules and other centriole-related structures in speed, orientation and persistence has been studied in the motile blood eosinophils of the newt, Tarchia granulosa, by Koonce, et.al.(Koonce, Cloney & Berns, 1984) during random migration. These cells have asymetric arrays of microtubules aligned along the axis of movement and normally migrate very persistently. When the centrisomal region of a moving cell was irradiated with a laser microbeam; that cell migrated subsequently at a slower speed and made more turns. When such cells were stained for tubulin, by imunofluorescence, the asymmetrical array of microtubules was missing, thus implicating the microtubules in the maintenance of persistence.

If the ability of neutrophils to polarize in a chemotactic gradient were altered during storage, the cell would

make turns at random, lose orientation and move more slowly because the contractile waves were less efficient. Such behavior characterizes a subpopulation of stored neutrophils.

2)Intermediate filaments

Neutrophils have vimentin intermediate filaments (Parysek. et.al., 1985). Several authors (Davis et al., 1982, Parysek, et.al., 1985) have reported that the intermediate filaments of neutrophils formed a tangled complex in the tail of cells during chemotactic locomotion. This structure could associate with the aligned microtubules and produce a resistive drag within the cytoplasm which would stabilize the orientation of the migrating cell and account for the characteristic uropod of migrating neutrophils.

Existence of subpopulations

The chemotactic behavior of some stored neutrophils is characterized by slower, less persistent, less accurate motion (Burton, et.al., 1985c). We now show that 1) these slower moving stored cells are a separate normally distributed population which can be distinguished mathematically from those cells moving at normal speed (fig. 5a,5b) and 2) the cells which show decreased orientation accuracy are part of a distinct population which is also present in fresh cells (figs 6a, 6b). The storage lesion appears to be a shift of cells between these pre-existing subpopulations.

The existence of subpopulations in randomly migrating

neutrophils has been shown (Elgefors, et.al., 1984, Howard, 1982). Bultmann, et al. (Bultmann, Haferkamp, Eggers & Gruler, 1984) analysed the N-fMLP stimulated chemotactic locomotion of normal neutrophils and neutrophils which had been infected with a virus (Echo 9 A.B.). Their results showed that the orientation of normal neutrophils was normally distributed. The distribution for the orientation of virus-infected cells was offset from the baseline. This could be accounted for by adding a constant to the Gaussian equation. The constant was attributed to the presence a subpopulation of cells which oriented randomly in the gradient.

We expanded on Bultmann's approach by optimizing our Gaussian equations using the chi-squared test of goodness-of-fit and showed that the equations for both fresh and stored neutrophils contain constants (fig 7a & 7b). This indicated that both fresh and stored populations had cells that oriented at random in a chemoattractant gradient. The magnitude of these constants (fresh: 0.09 vs stored 0.35) is indicative of the relative sizes of the non-chemotactic populations.

Conclusions

Neutrophils exist in two motile states which can be distinguished by their ability to orient in a chemotactic gradient. This behavior may be the result of the organization of the cytoskeleton and the biochemical processes which

regulate its structure. Storage at 20-22° C, induces a change in the "steering system" and "intracellular compass" such that the information in the gradient is not tranduced effectively into directed locomotion.

Many (35%) of the stored cells show slower, randomly oriented locomotion in response to a chemotactic gradient which is qualitatively similar to chemokinesis. The chemokinetic speed, persistence and turning behavior of neutrophils has been thoroughly characterised (Keller, 1985a,b, Burton, et.al., submitted for publication). In a companion paper (Burton, Bank & Law, In preparation), We propose a model to explain the the relationship between chemotactic and chemokinetic locomotion.

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FIGURE LEGENDS

Fig 1. Histogram of speed of fresh and stored neutrophils.

Fig 2. Histogram of persistence index of fresh and stored neutrophils.

Fig 3. Histogram of orientation angle of fresh and stored neutrophils.

Fig 4. Histogram of chemotropic index of fresh and stored neutrophils.

Fig 5a. Cumulative frequency distribution of speed of stored neutrophils. Note departure from expected Gaussian cureve in the ~3-12 um/min range.

Fig 5b Cumulative freq. distribution of 3-12um/min range of speed of stored cells. A new Gaussian curve was calculated from the mean and standard deviation of the cells in this speed range.

Fig 6 Frequency distribution of orientation for neutrophils with a Gaussian curve with the same mean and standard deviation superimposed, observed [- - -], expected [...], a) fresh, b) stored. Note that the distribution of the data for stored cells is offset above the baseline.

Fig 7 Frequency distribution of orientation with a Gaussian curve fitted to the data by Chi-squared optimization, observed[- - -], expected [...], a. fresh cells, b. stored cells. Note that the standard deviations for both fresh and stored are the same and that constants account for the offset of the curves above the baseline.

Fig 8. Scatter-diagrams of speed vs. persistence index for a) fresh and b) stored neutrophils.

Fig 9 Scatter-diagrams of speed vs. orientation angle for a) fresh and b)stored neutrophils.

Fig 10 Scatter-diagrams of speed vs chemotropic index for a) fresh and b) stored neutrophils.

Fig 11 Scatter-diagrams of orientation angle vs persistence index for a) fresh and b) stored neutrophils.

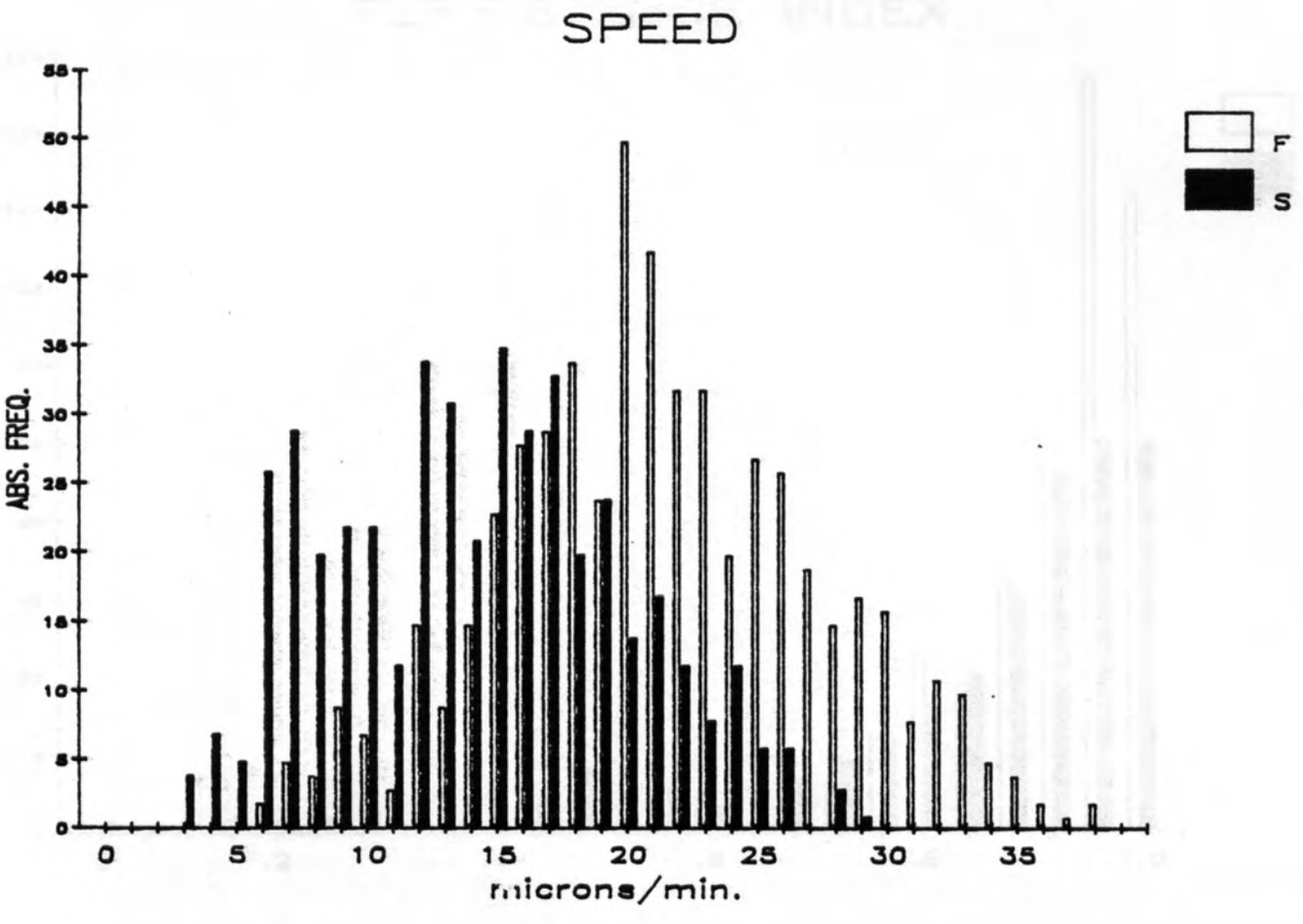
Fig 12 Scatter-diagrams of persistence index vs. chemotropic index for a) fresh and b) stored neutrophils.

Fig 13 3-D histograms of speed vs. persistence index for a) fresh and b) stored cells.

Fig 14 3-D histograms of speed vs. chemotropic index for a) fresh and b) stored cells.

Fig 15 3-D histograms of persistence index vs. chemotropic index for a) fresh and b) stored cells.

FIGURE 1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5
10
15
20
25
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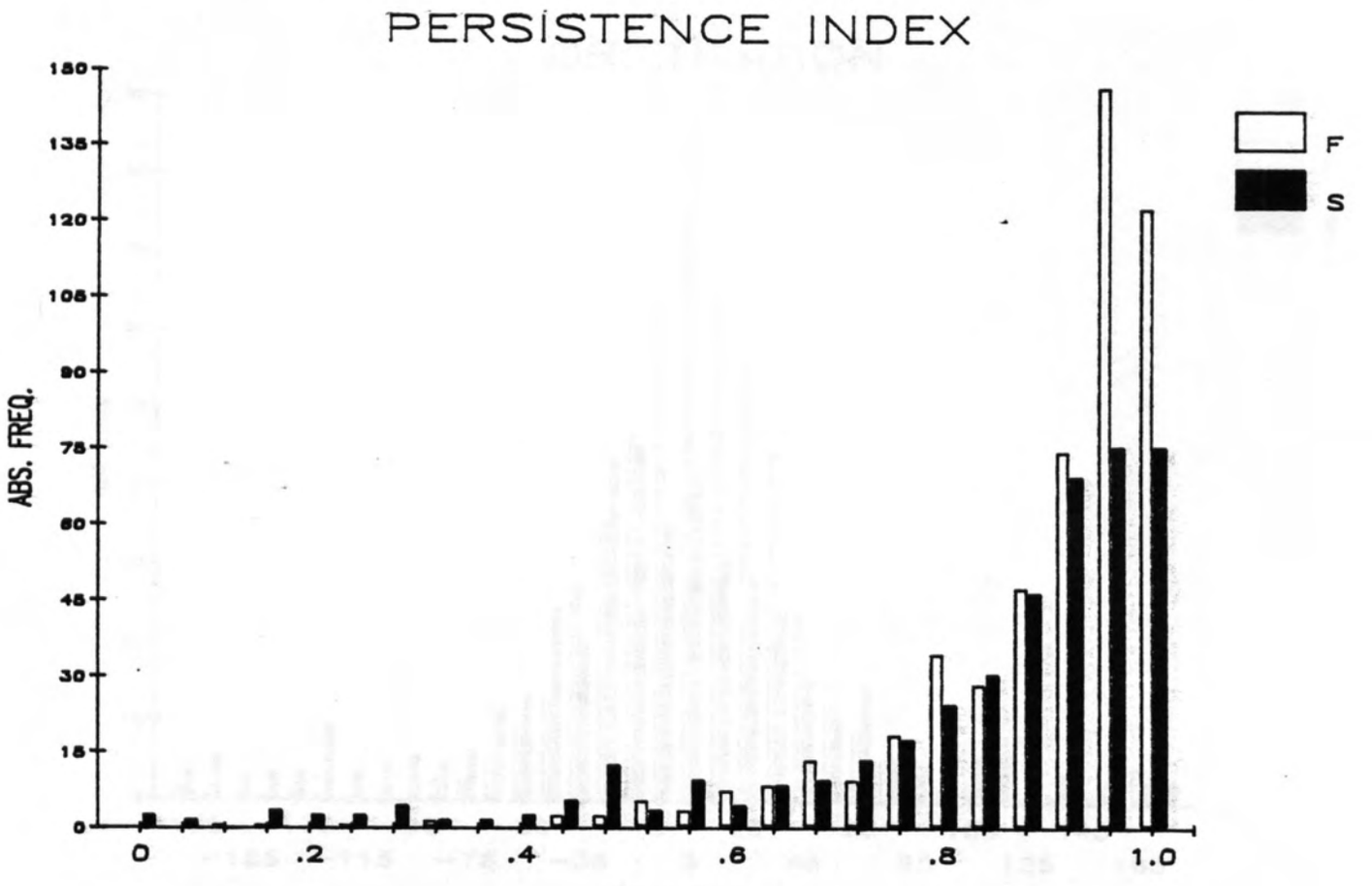
15
20
25
30
35
microns/min.
F
S

FIGURE 2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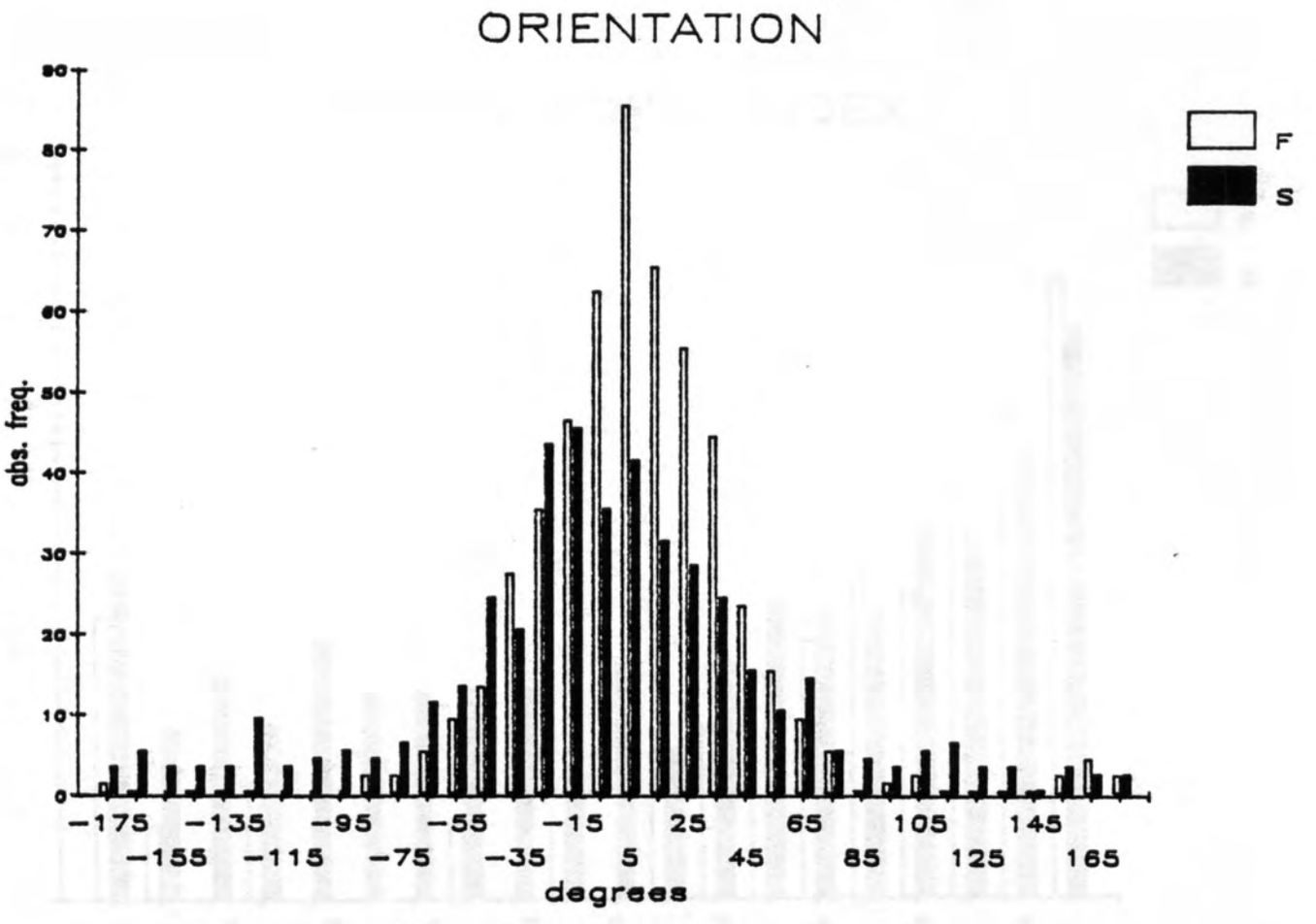
.8
1.0
F
S

FIGURE 3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S
abs. freq.
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10
20
30
40
50
60
70
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90
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−135
−115
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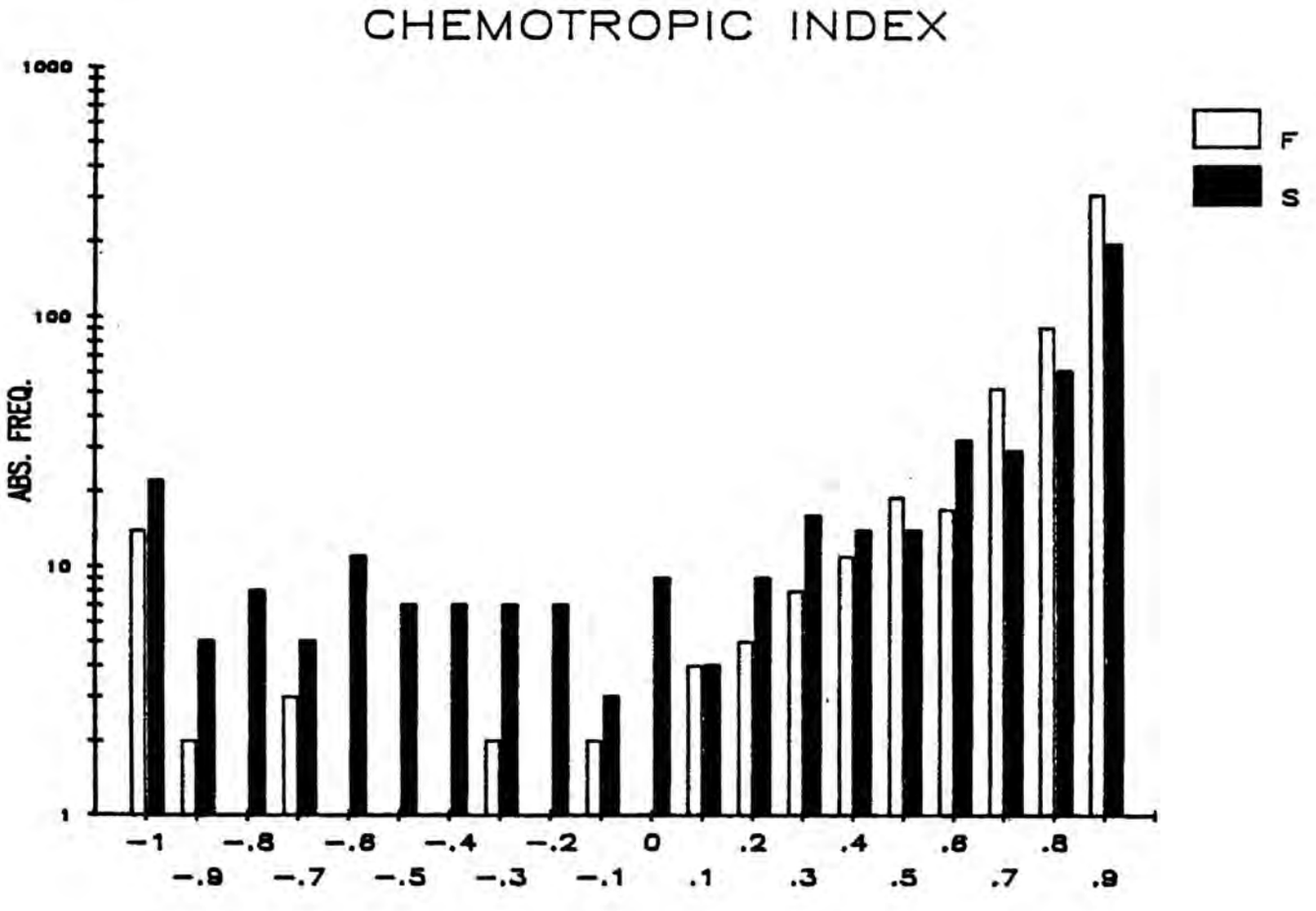
5
25
45
65
85
105
125
145
165
degrees

FIGURE 4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−.8
−.7
−.6
−.5
−.4
−.3
−.2
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.4
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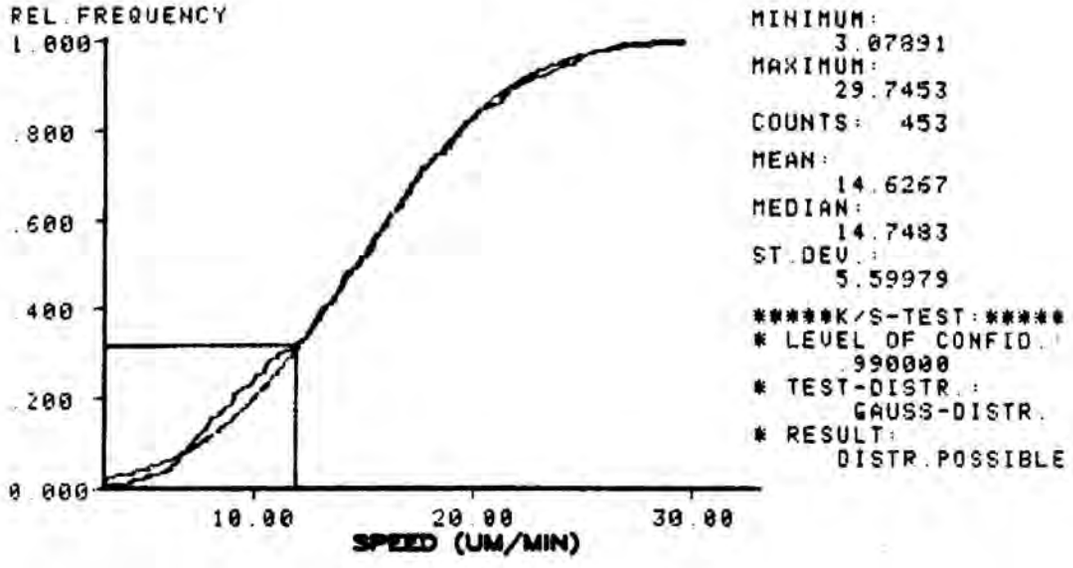
.6
.7
.8
.9

FIGURE 5a-b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.800
.600
.400
.200
0.000
10.00
20.00
30.00
SPEED (UM/MIN)
MINIMUM:
3.07891
MAXIMUM:
29.7453
COUNTS: 453
MEAN:
14.6267
MEDIAN:
14.7483
ST.DEV.:
5.59979
*****K/S-TEST:*****
* LEVEL OF CONFID.:
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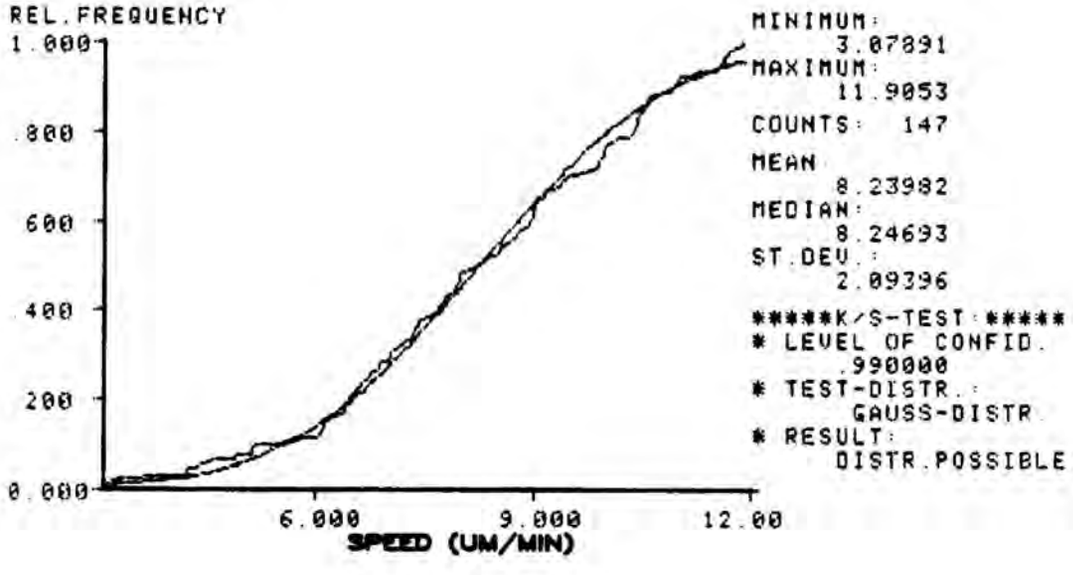
* TEST-DISTR.:
GAUSS-DISTR.
* RESULT:
DISTR.POSSIBLE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9.000
12.00
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MAXIMUM:
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COUNTS: 147
MEAN
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MEDIAN:
8.24693
ST.DEV.:
2.09396
*****K/S-TEST:*****
* LEVEL OF CONFID.
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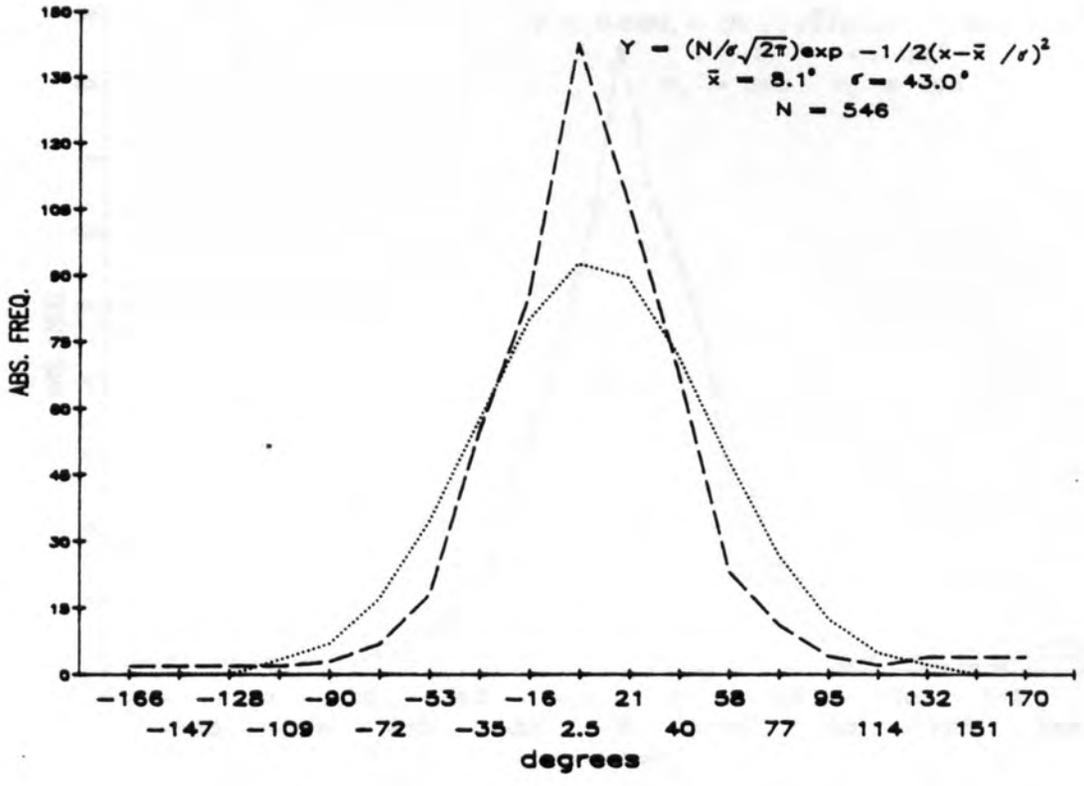
* TEST-DISTR.:
GAUSS-DISTR
* RESULT:
DISTR.POSSIBLE

FIGURE 6a-b


Y = (N/σ√2π)exp −1/2(x−x̄ /σ)²
x̄ = 8.1° σ = 43.0°
N = 546
ABS. FREQ.
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15
30
45
60
75
90
105
120
135
150
−166 −128 −90 −53 −16 21 58 95 132 170
−147 −109 −72 −35 2.5 40 77 114 151
degrees

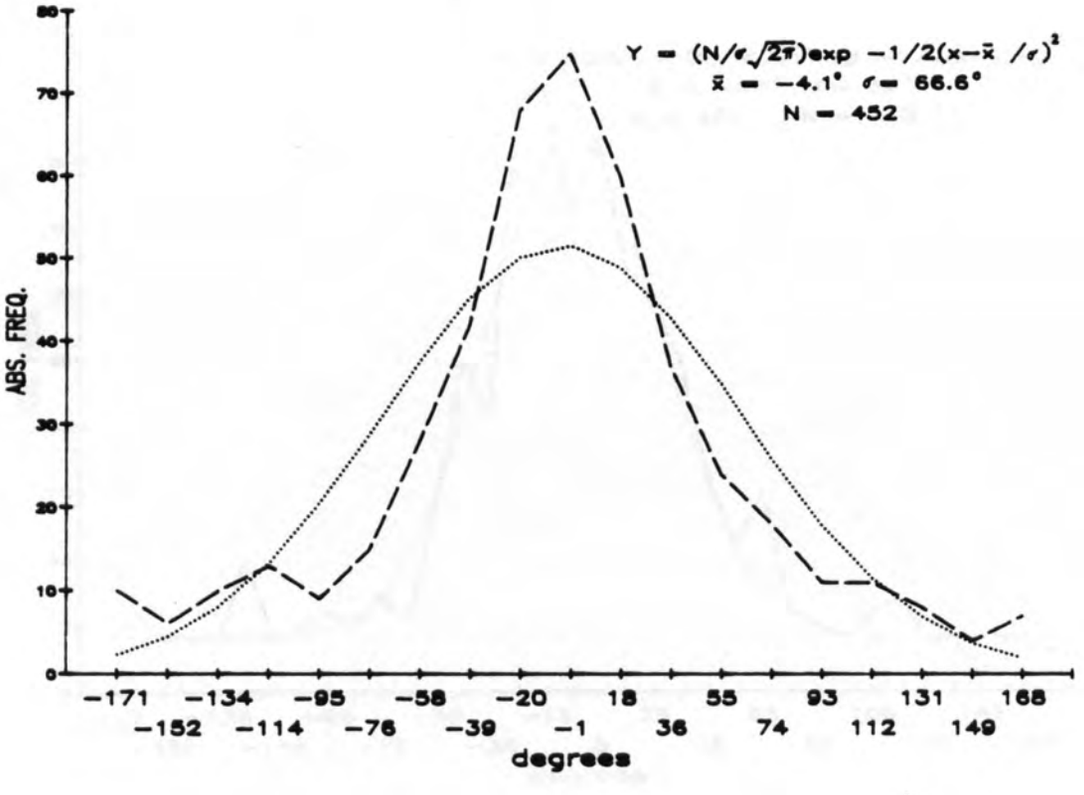
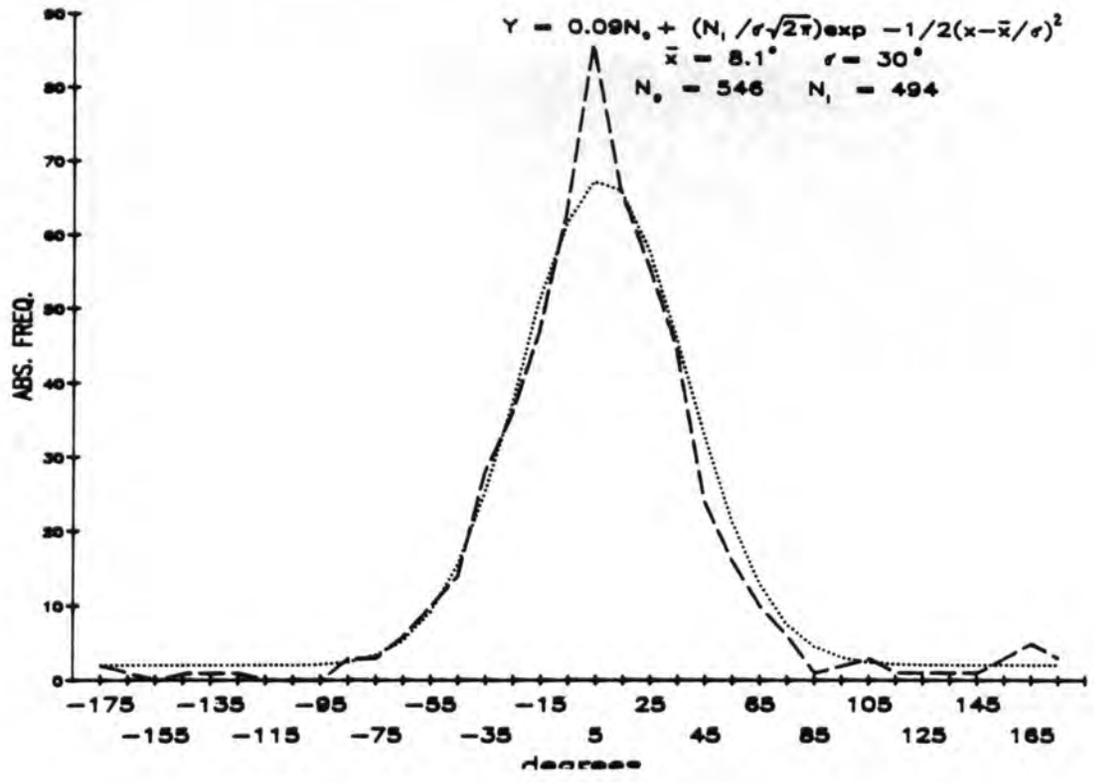
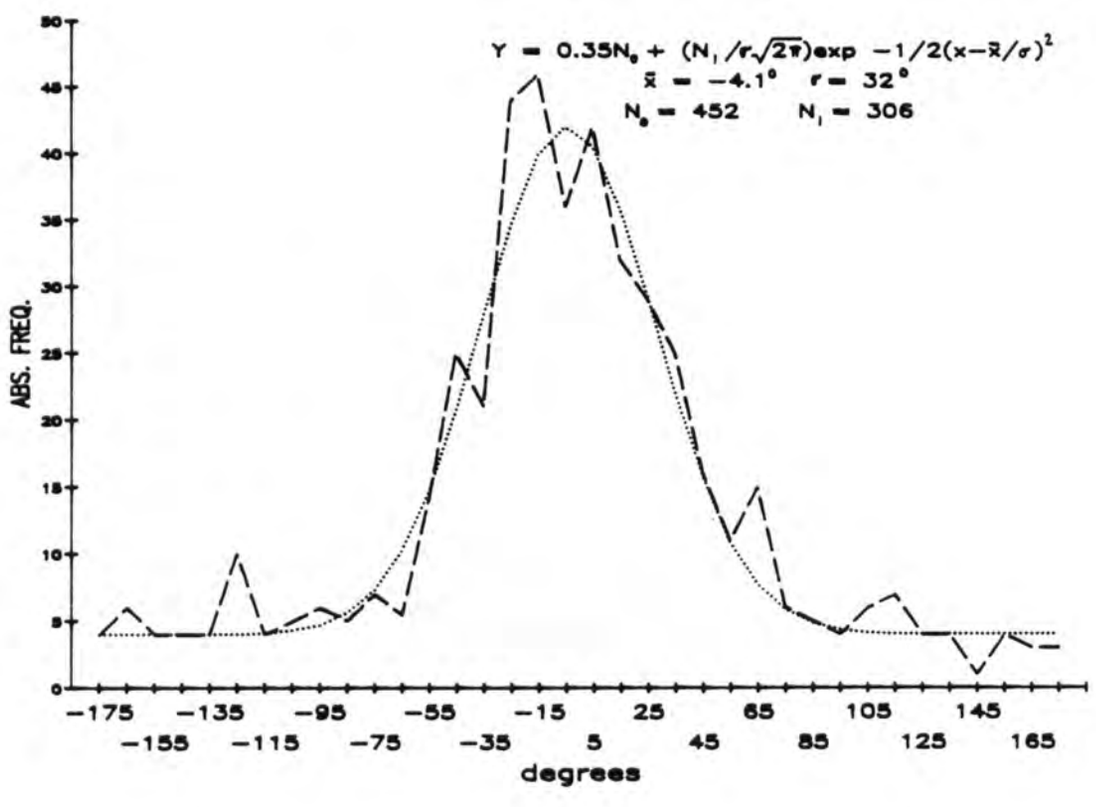

Y = (N/σ√2π)exp −1/2(x−x̄ /σ)²
x̄ = −4.1° σ = 66.6°
N = 452
ABS. FREQ.
0
10
20
30
40
50
60
70
80
−171 −134 −95 −58 −20 18 55 93 131 168
−152 −114 −76 −39 −1 36 74 112 149
degrees

FIGURE 7a-b


Y = 0.09N₀ + (N₁ /σ√2π)exp −1/2(x−x̄/σ)²
x̄ = 8.1° σ = 30°
N₀ = 546 N₁ = 494
ABS. FREQ.
−175 −135 −95 −55 −15 25 65 105 145
−155 −115 −75 −35 5 45 85 125 165


Y = 0.35N₀ + (N₁ /σ√2π)exp −1/2(x−x̄/σ)²
x̄ = −4.1° σ = 32°
N₀ = 452 N₁ = 306
ABS. FREQ.
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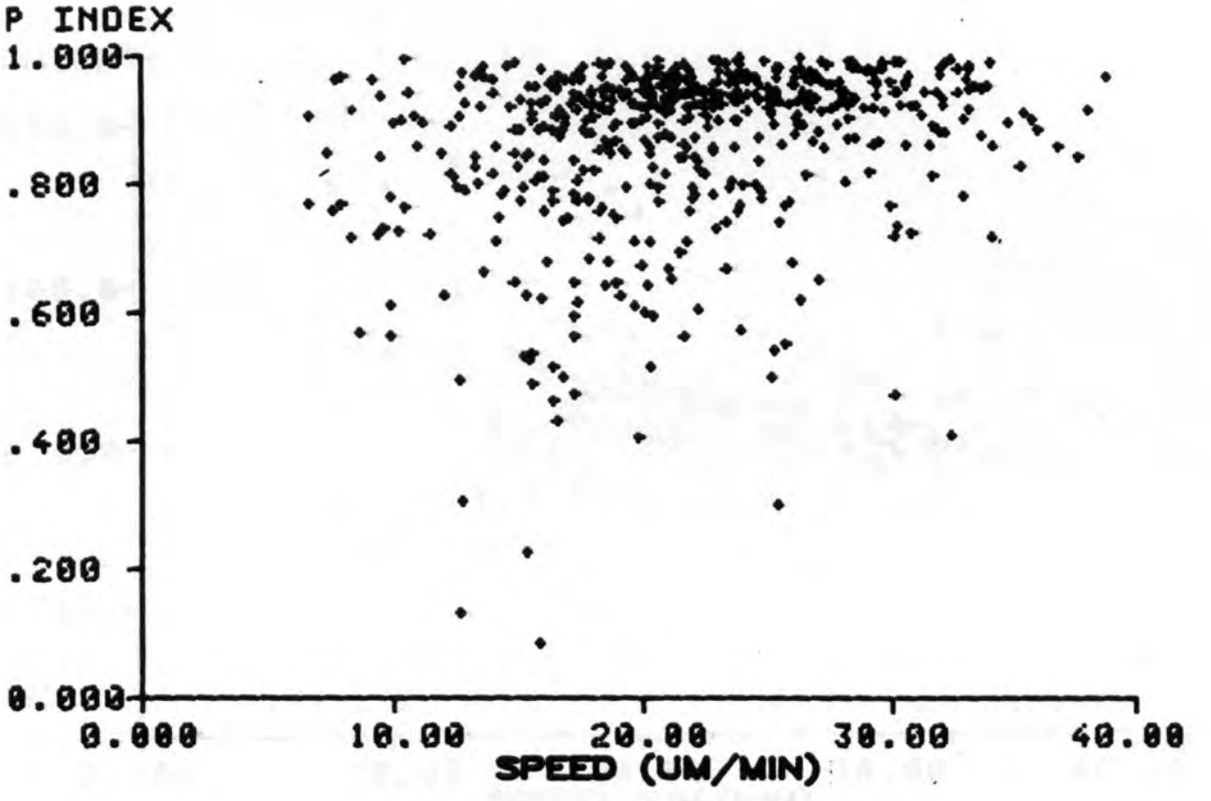
−155 −115 −75 −35 5 45 85 125 165
degrees

FIGURE 8a-b


P INDEX
1.000
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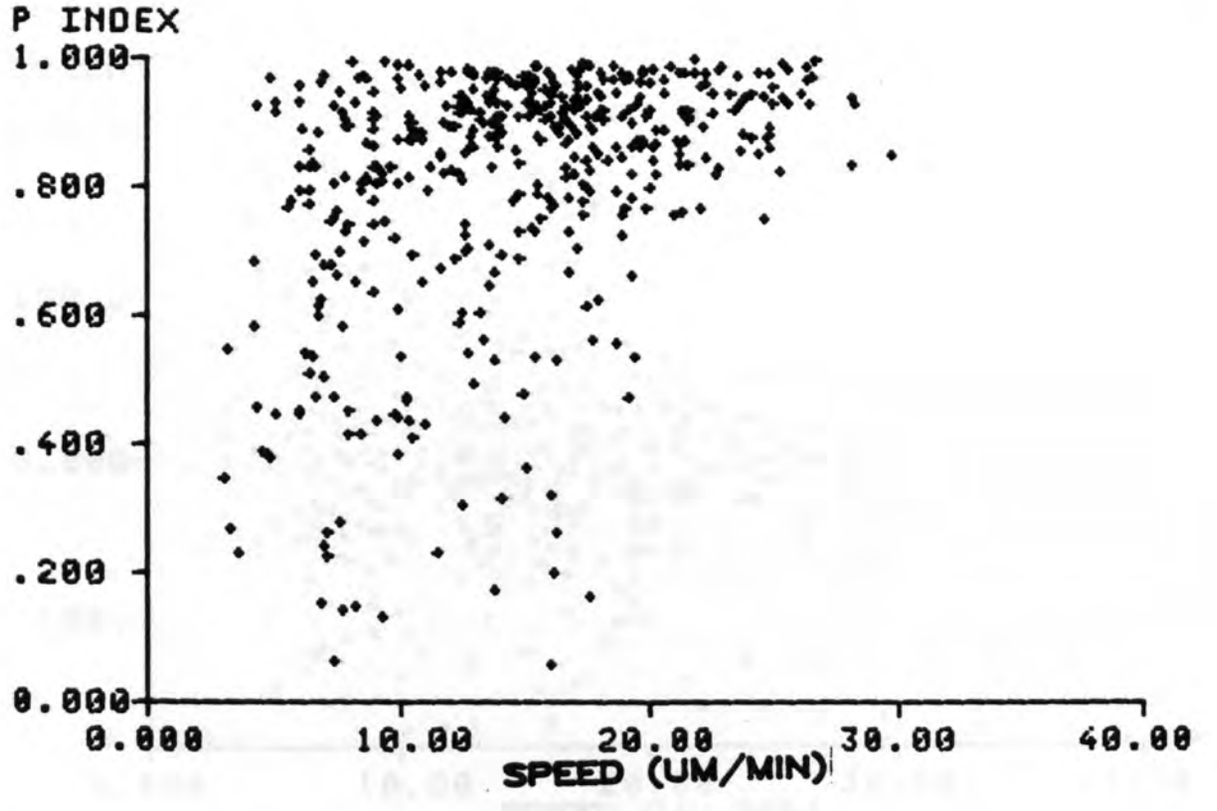
.600
.400
.200
0.000
0.000
10.00
20.00
30.00
40.00
SPEED (UM/MIN)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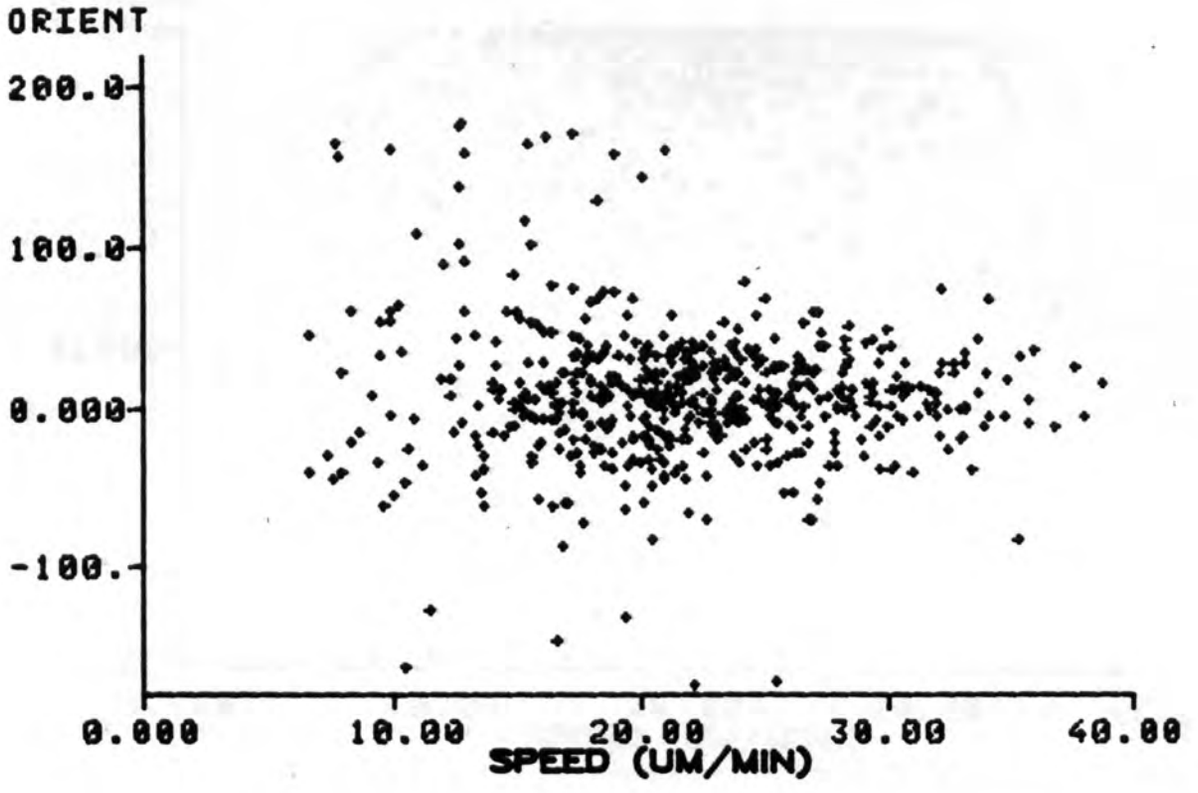
SPEED (UM/MIN)

FIGURE 9a-b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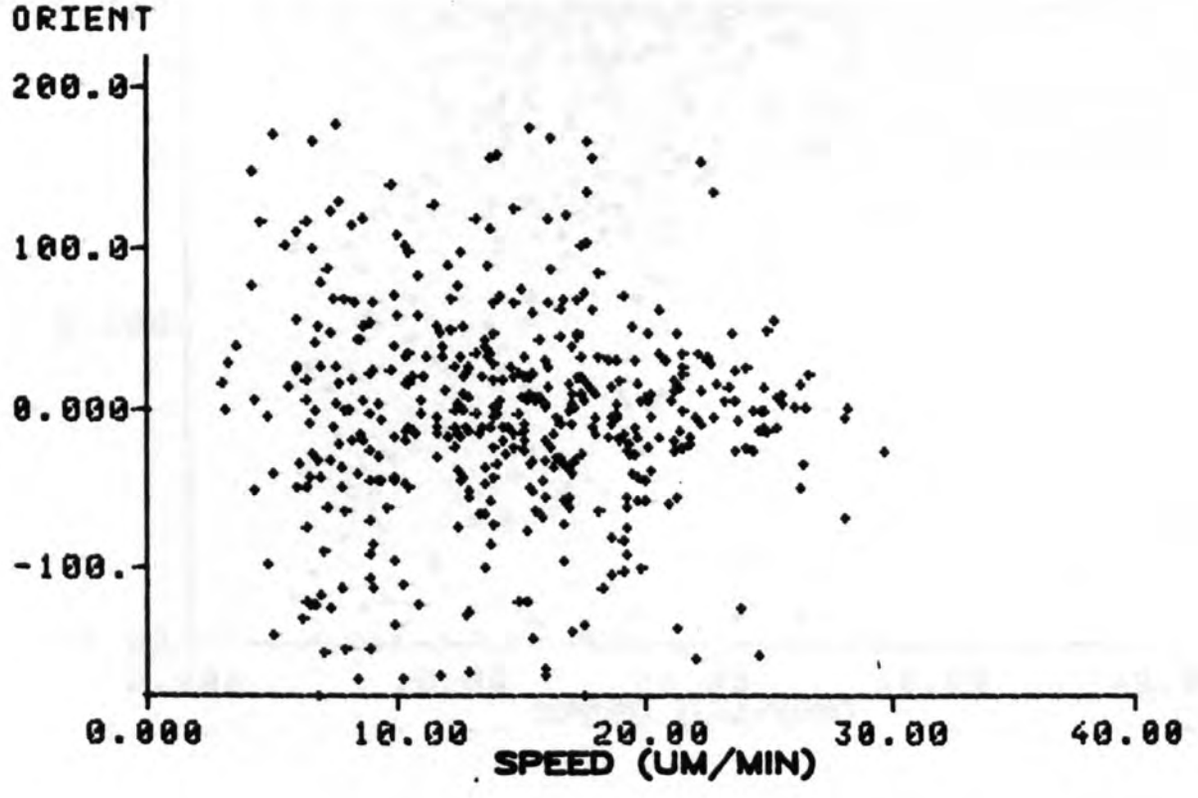
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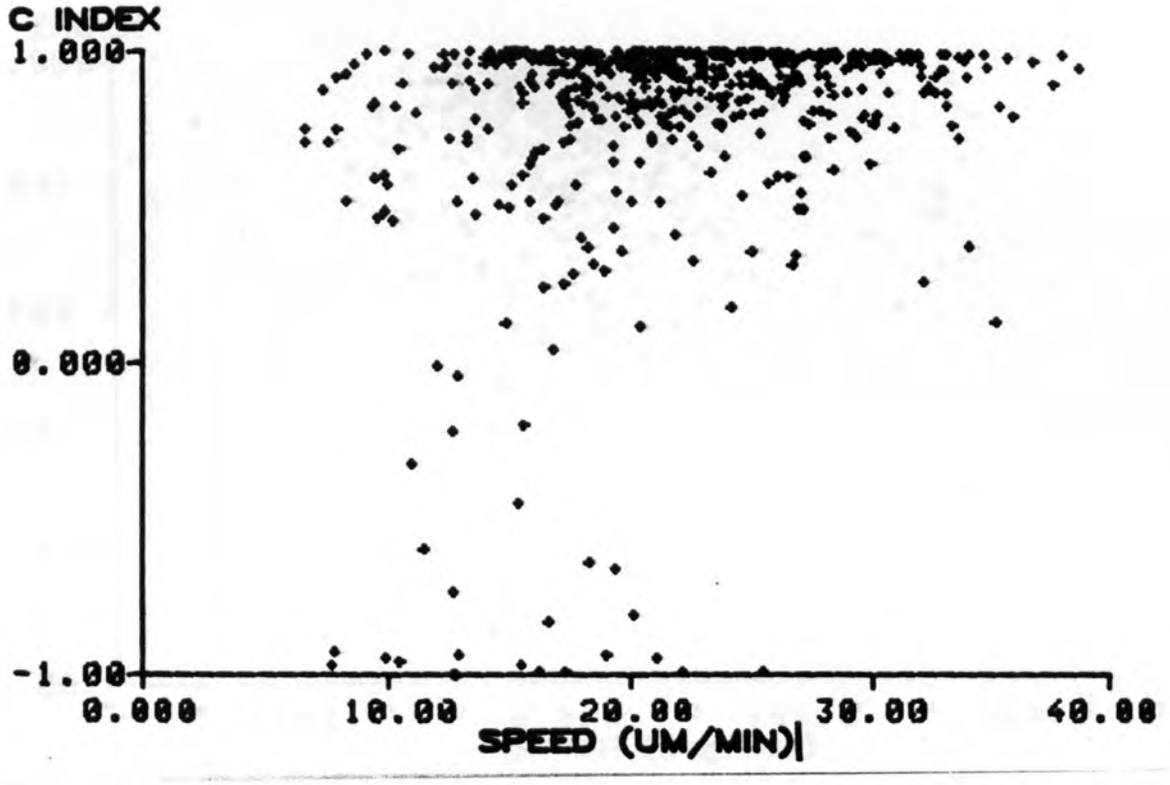
SPEED (UM/MIN)

FIGURE 10a-b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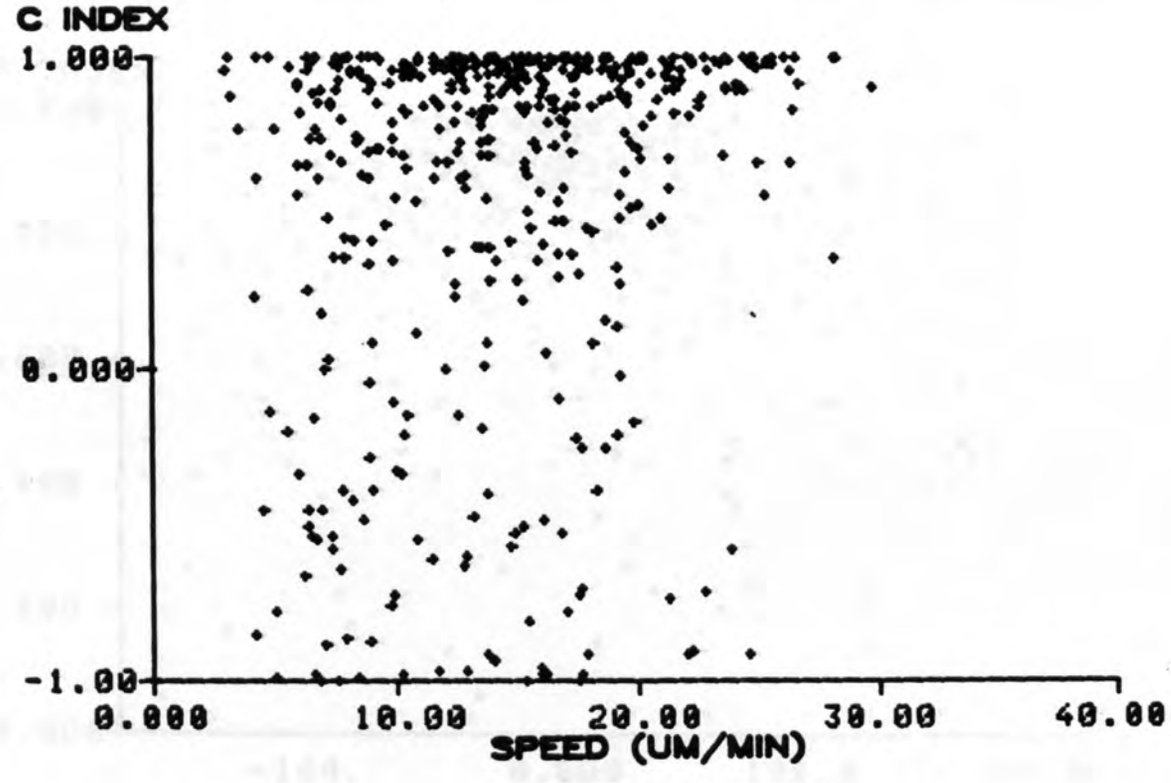
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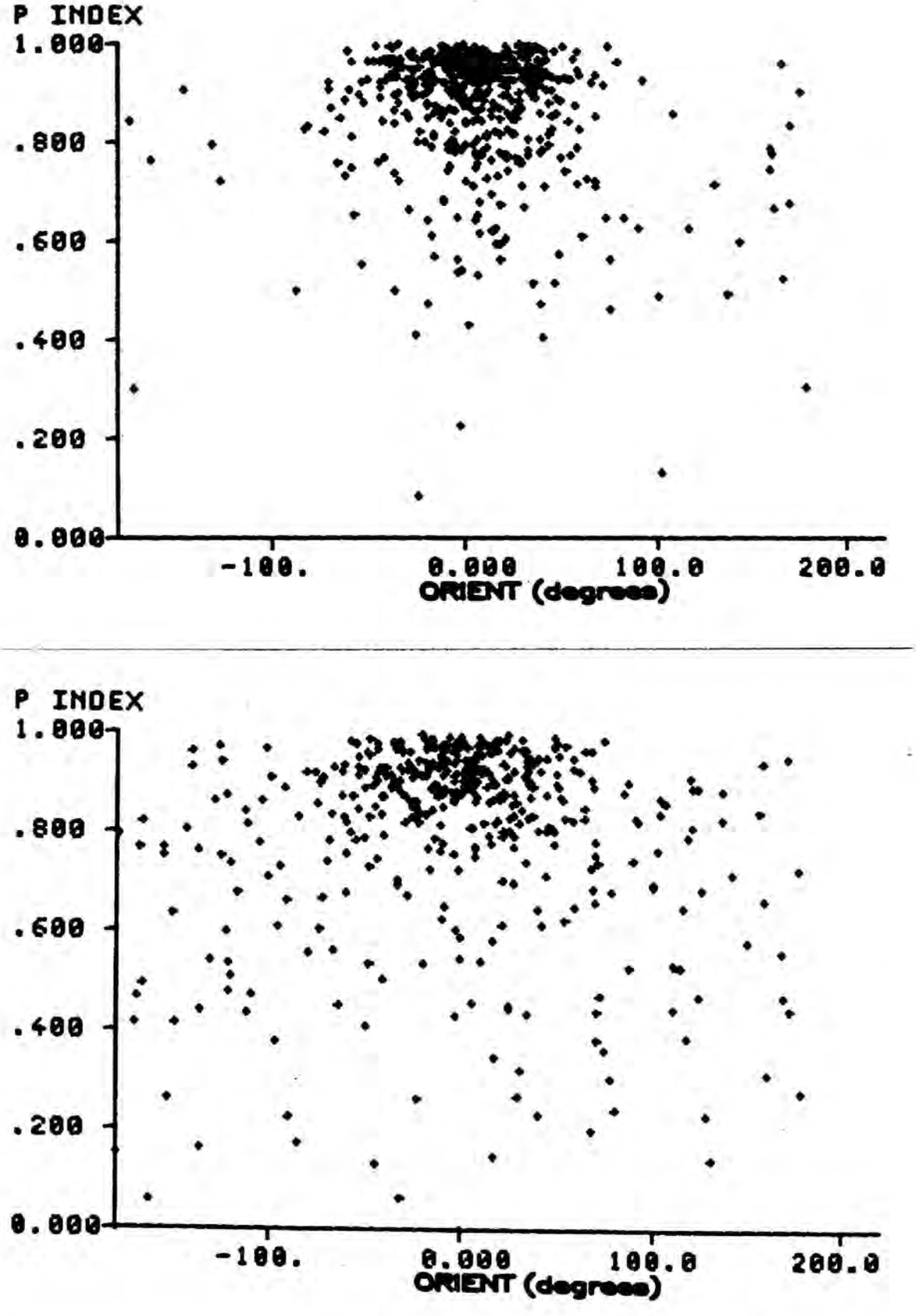
SPEED (UM/MIN)

FIGURE 11a-b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0.000
100.0
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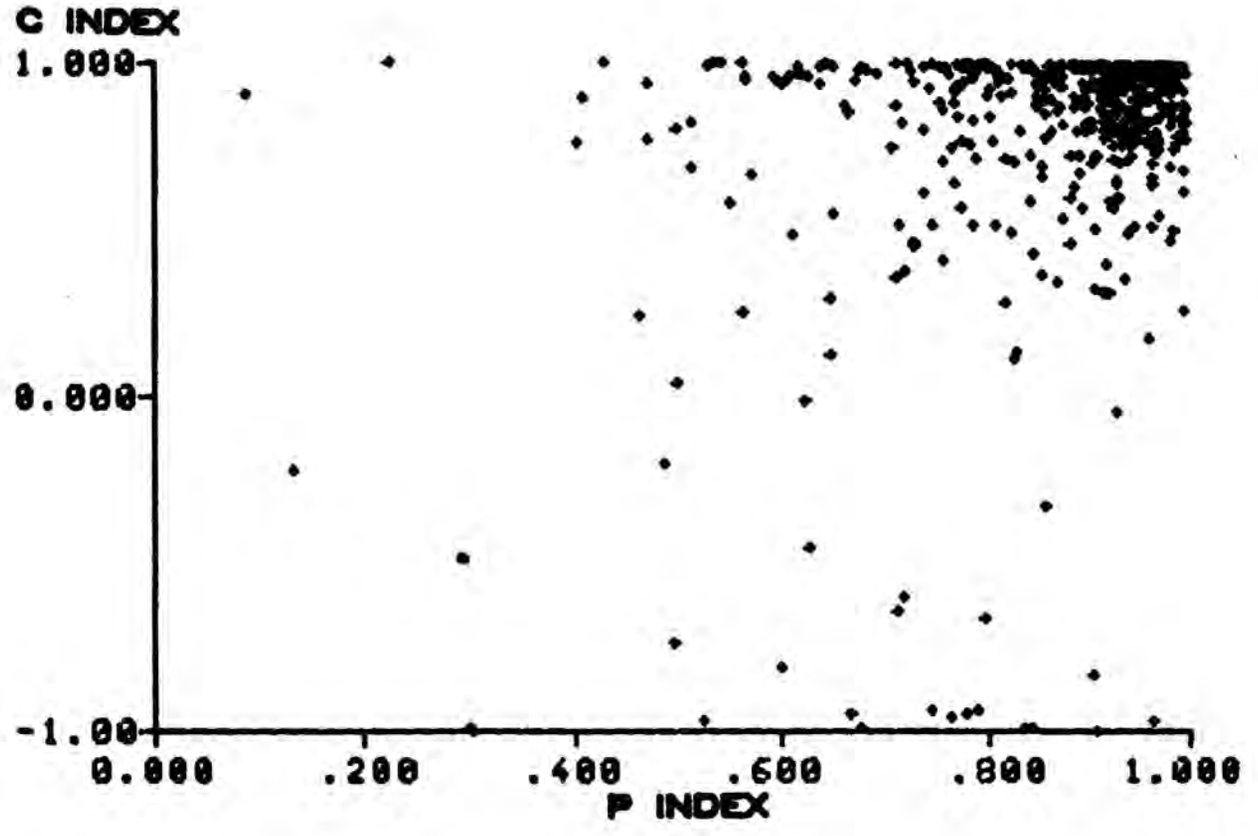
ORIENT (degrees)

FIGURE 12a-b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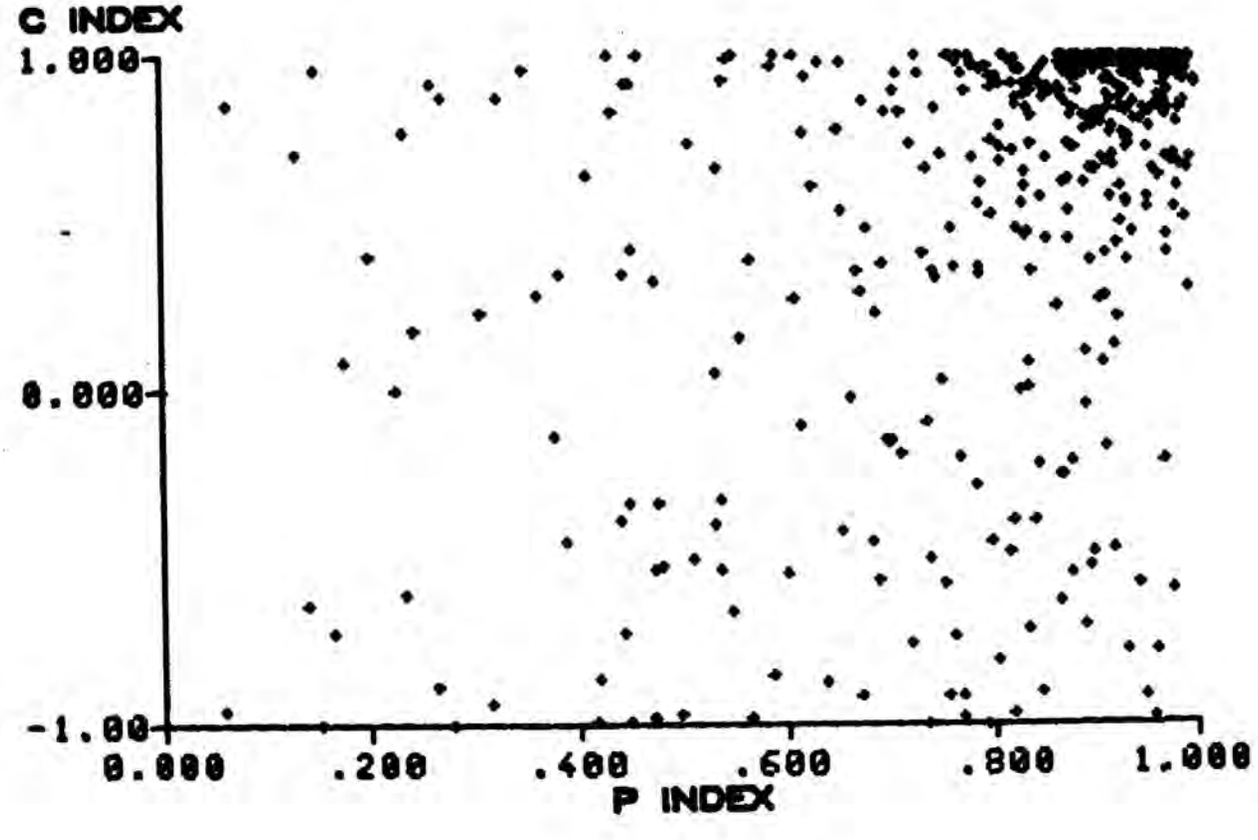
P INDEX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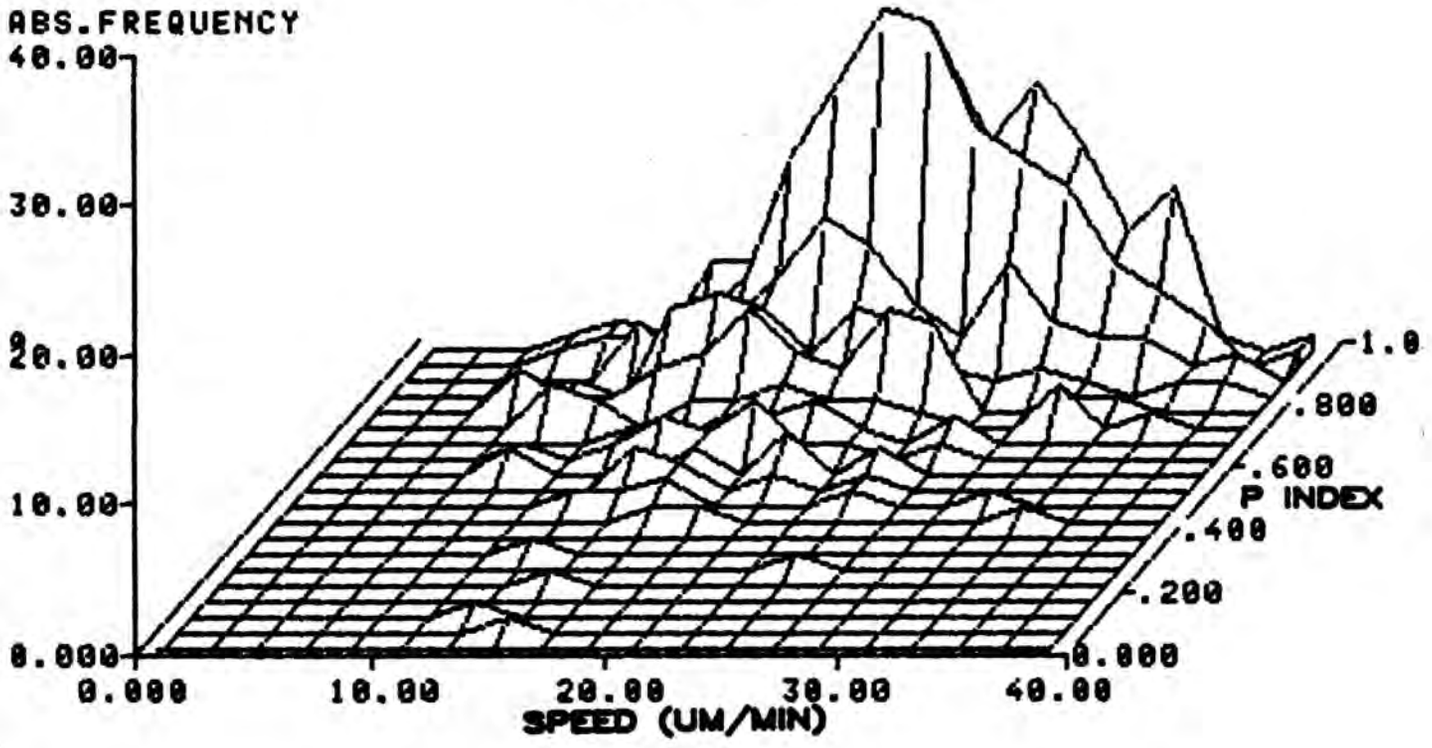
P INDEX

FIGURE 13a-b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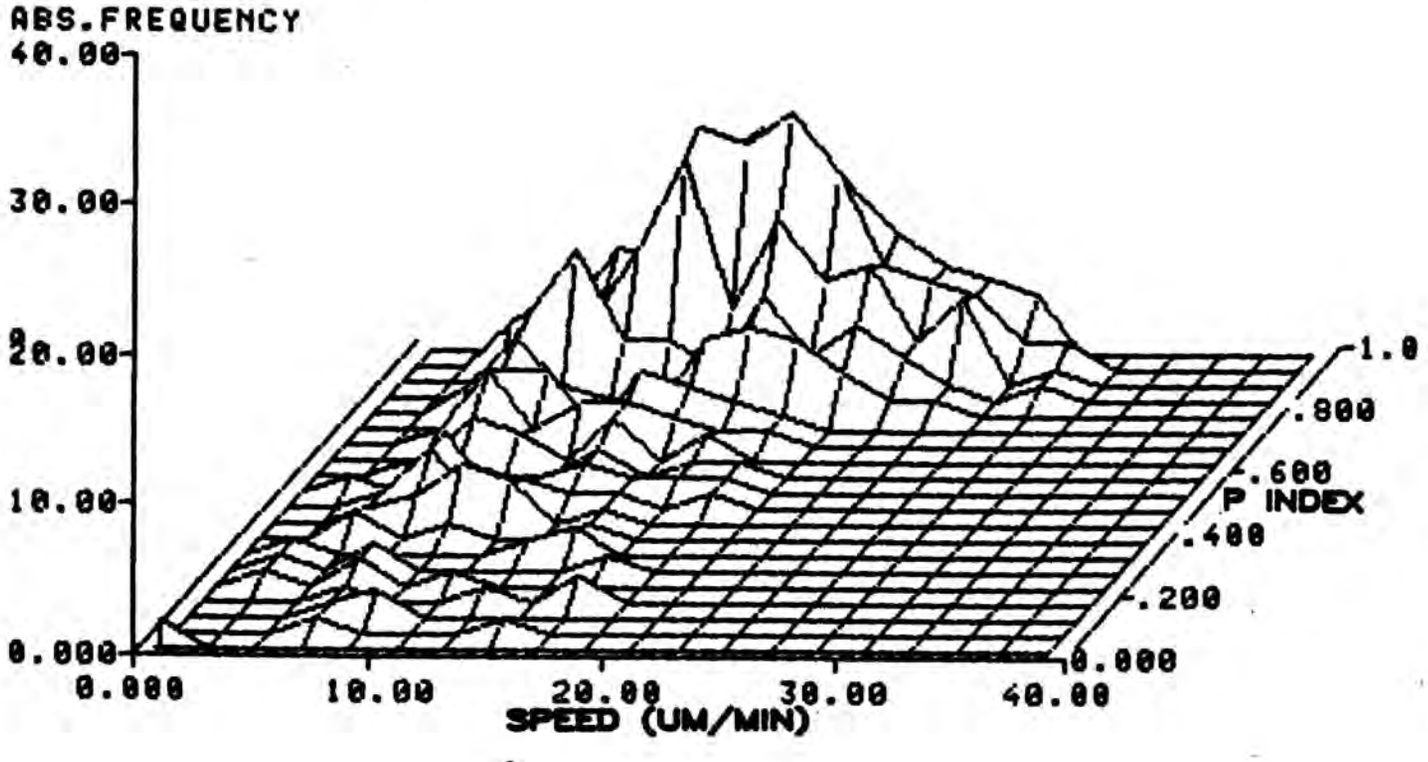
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P INDEX
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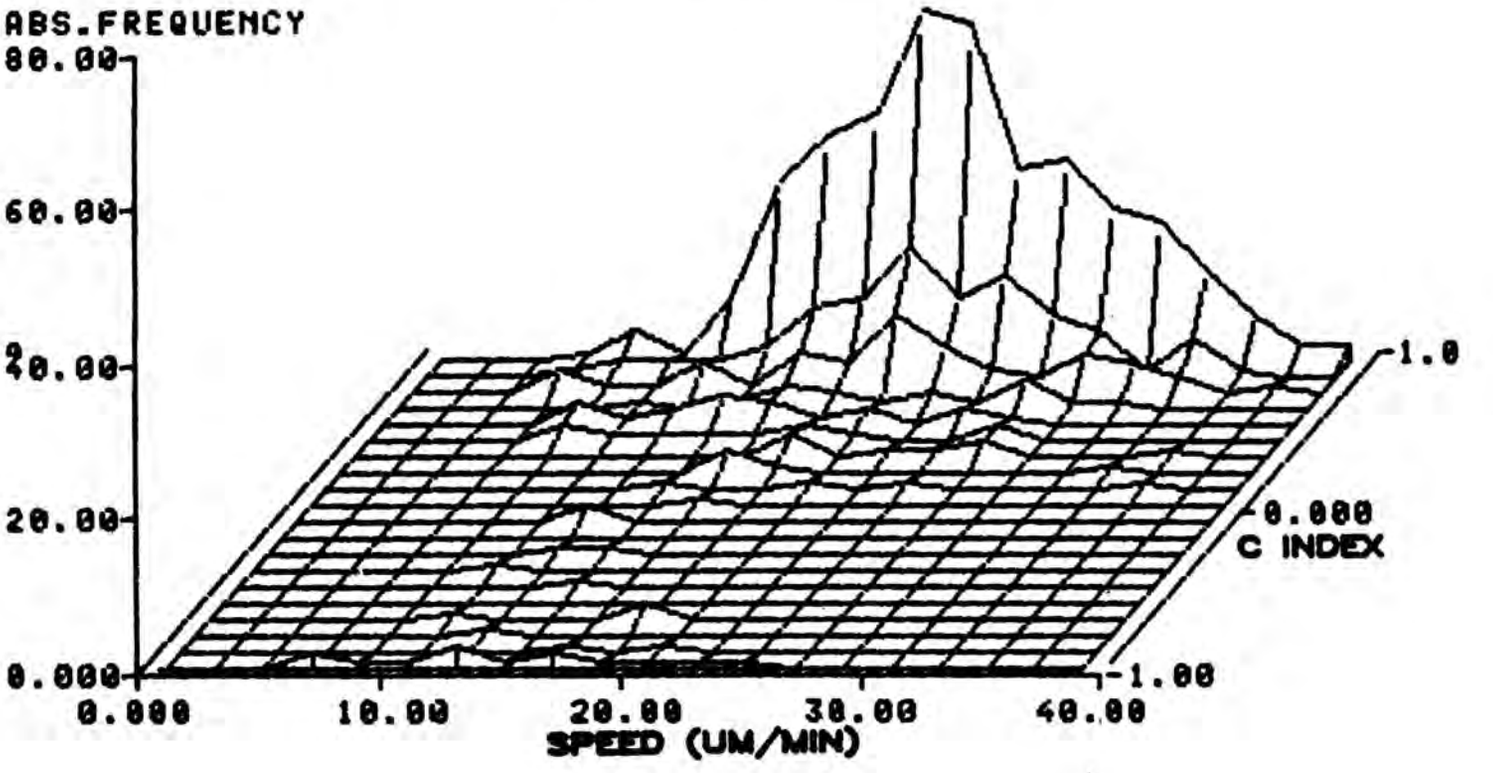
FIGURE 14a-b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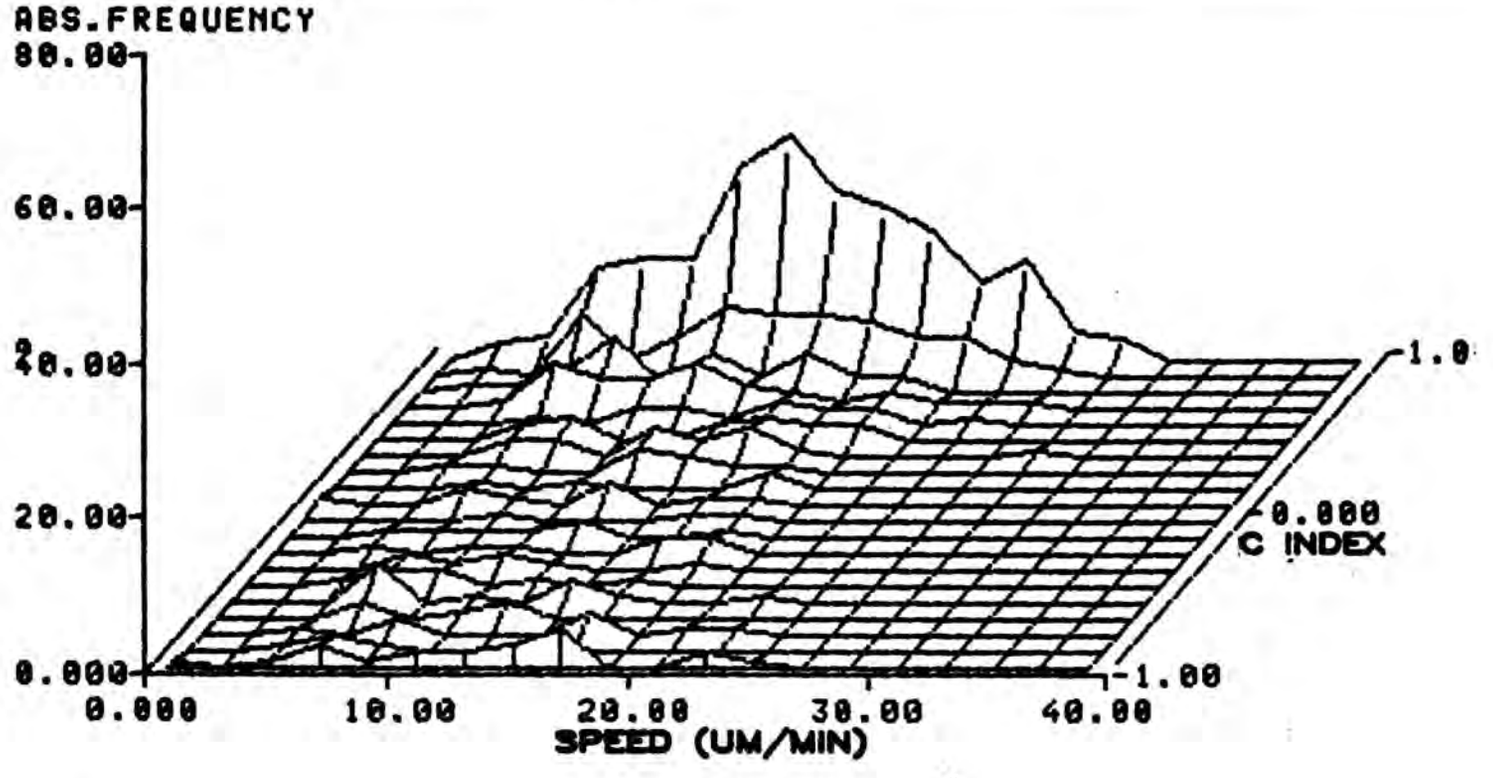
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SPEED (UM/MIN)
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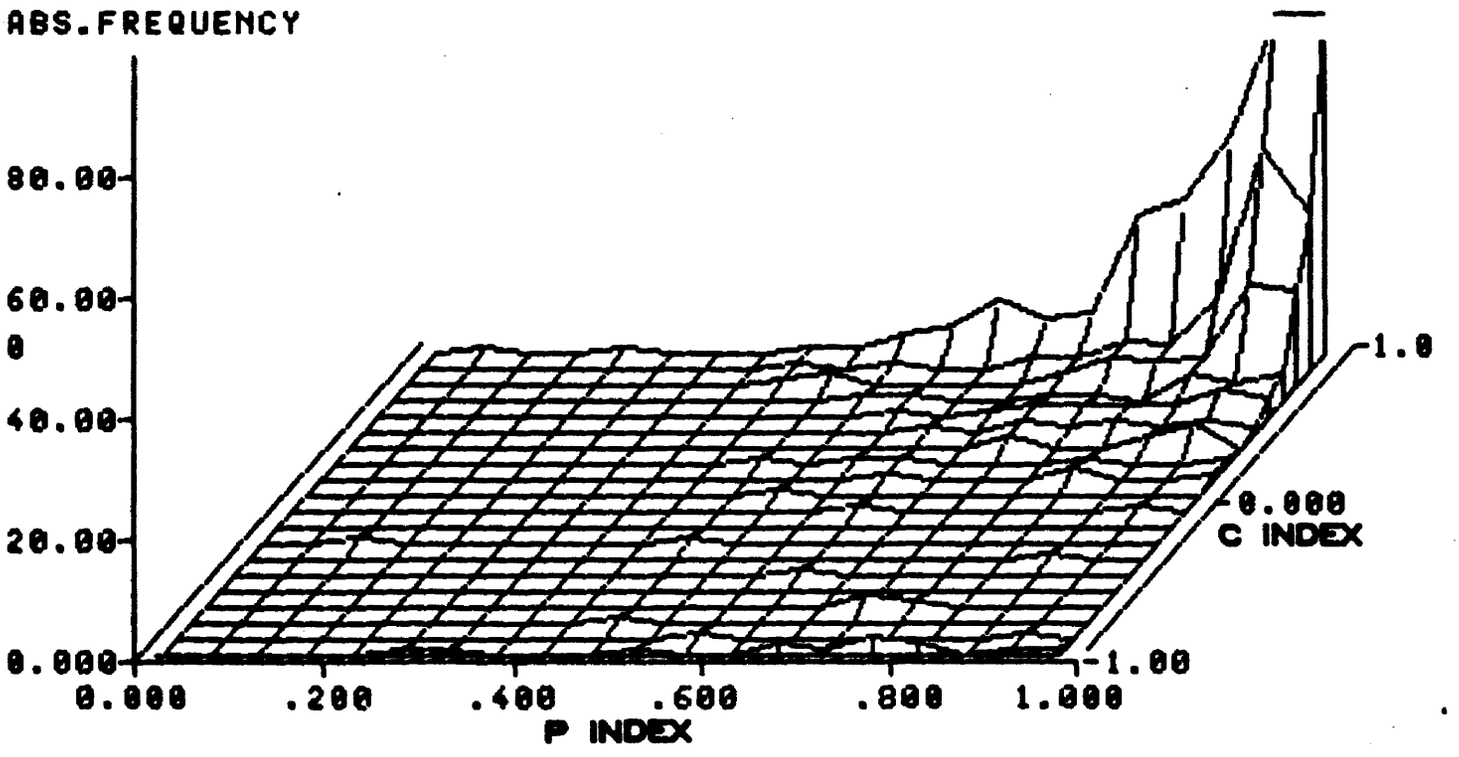
C INDEX
-1.00

FIGURE 15a-b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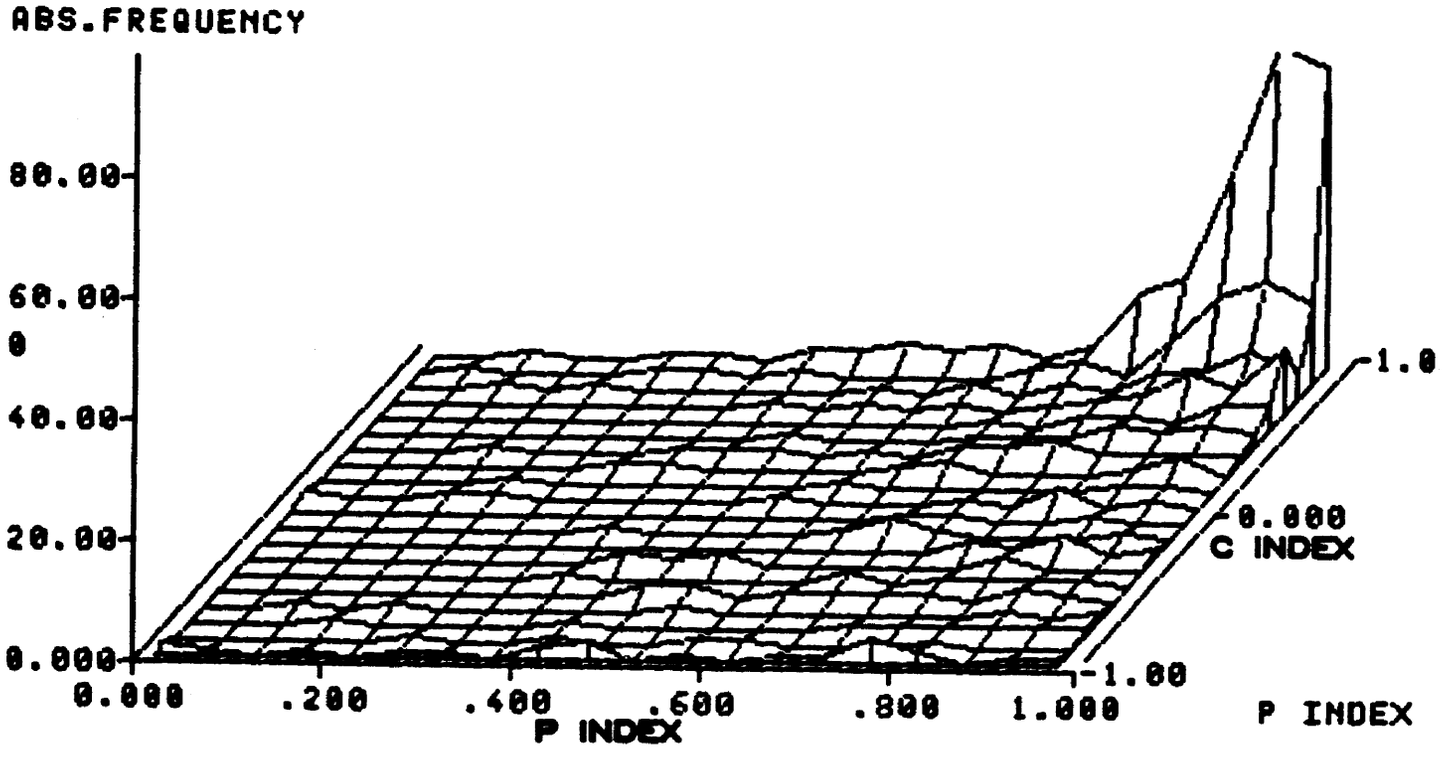
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CHAPTER VII.

CONCLUSIONS AND A MODEL FOR THE RELATIONSHIP BETWEEN CHEMOKINESIS AND CHEMOTAXIS

The results of the studies described in Chapts. 4,5 and 6 support the concept that chemokinesis and chemotaxis are modes of behavior which represent distinct intrinsic motile states of the neutrophil. When freshly isolated neutrophils are stimulated by a gradient of chemoattractant ligand, the vast majority of cells which are capable of responding to the ligand, orient accurately along the gradient and migrate toward the source in a highly persistent manner (make few/ narrow turns). A small fraction (~10%) of the cells capable of migration cannot use the information in the gradient and move at a much slower speed without any stable orientation, making more/ wider turns as they proceed. When neutrophils are stored at room temperature for 24 hr., under conditions which prevent cell death due to depletion of substrate, more cells become non-responders and some cells shift to a state where their response to the gradient of ligand is quantitatively comparable to normal chemokinesis. The alteration induced by storage appears to involve a "switch" between the two motile states.

Fig. 1 is a flowchart which diagrams the proposed sequence of events which describe chemokinesis and chemotaxis. When an unstimulated cell is exposed to a chemoattractant and binding of the ligand to the specific receptor on the membrane occurs. If the cell is capable of responding, a

series of events causes the cell to assume a "polarized" configuration and contractile waves, initiated in the lamellipod, begin to propagate along the length of the cell. If the cell is in contact with a substrate and the distribution of the ligand is isotropic, the cell will adhere to the substrate and migrate at a characteristic speed and maintain directional persistence. This mode of migration persists until, by chance, the source of the ligand is located. If the ligand source is a particle which can be phagocytosed, the cell will engulf the particle. If the stimulation of the cells receptors induces secretion of the primary granules (degranulation) the cell is rendered refractory to further stimulation and stops.

When a gradient of ligand is present, those cells capable of responding to that gradient will migrate at a higher mean speed, orient toward the source with high accuracy and show very high directional persistence. This rapid, accurate migration is characteristic of chemotaxis. These cells continue migrating toward the source until they receive "maximal" stimulation and degranulate. The maximal stimulation may involve phagocytosis of target particles or encountering a concentration of the ligand which is sufficiently high to induce degranulation. Either way the cell then stops. Even in the presence of a ligand gradient, some cells do not transduce the information in the gradient and are only capable of the kind of locomotion characteristic

of chemokinesis.

Storage affects the "switch" between the two motile states such that more stored cells respond to the gradient with chemokinesis-like locomotion (slower speed, lower directional persistence and no prefered orientation) than fresh.

This model can also provide a framework for organizing the available data on stimulus-response coupling in neutrophils. The numbers in Fig. 1 indicate points where the sequence can be modulated.

1. If neutrophils are exposured to a high concentration of ligand, the affinty of the receptors decreases and subsequent exposure to ligand will not produce migration but rather cause secretion (Donabedian & Gallin, 1981). Likewise, if the cell is exposed to an agent which lowers the affinty of the receptor directly (ie., Amphotericin B), the cell will degranulate in response to the ligand (Lohr and Snydermann, 1982).

2. The second messenger for the locomotor system is probably calcium ions (Hartwig, et.al., 1983). Any agent which blocks or alters calcium homeostasis or inhibits calmodulin will block the response sequence at this point. This step is the probable control point which distinguishes responding cells from non-responding cells: cells which bind ligand normally, but do not migrate (Harvath and Leonard, 1982).

3. The mechanism for transduction of the information in the gradient of ligand into a structural asymetry in response to the gradient, is not known. This process is apparently altered during storage since loss of chemotaxis by stored cells seems to involve loss of the ability to transduce the information in the gradient. Possible cellular defects could involve altered calcium release, intracellular pH or membrane depolarization. In fully responsive cells, some localized cellular response to the gradient produces an asymetry or gradient inside the cell.

4. The set of responses which characterise chemokinesis involves the actin-myosin system which produces motion and some degree of structural asymmetry which is responsible for the directional persistence. One likely candidate is the intermediate filaments which are located in the posterior of migrating cells (Chapt. 6). The intermediate filaments are associated with coated pits which are also localized in the tail (Davis, et.al., 1982). Weinbaum and coworkers (1983) have reported that occupied peptide receptors cap on the rear of migrating cells, and are endocytosed. New receptors apppear preferentially at the leading edge of migrating cells. The rear of the cell is rendered less sensitive to stimulation due to this active directional displacement of receptor over the cell surface. The probability that an actin-based contraction of the cell cortex would be initiated by a hit of a receptor on the tail is reduced.

5.Since the speed and persistence in chemokinesis are lower than that of chemotaxis, some further change in structure must be triggered by the response to a gradient. Some other process or structure is necessary to impose additonal order on the cytoplasm before the contractile "motor" can operate with maximum efficiency. The increased motor efficiency which produces the higher chemotactic speed is probably a result of additional directonal stability in the moving cell.

The role of microtubules in chemotaxis has been the source of much debate (Zigmond, et. al., 1981) since colchicine-treated cells can still orient toward and migrate to a target particle (Allan & Wilkinson, 1978). Microtubules are probably involved in turning behavior rather than orientation because such colchicine-treated cells also make more/wider turns as they migrate (Allan & Wilkinson, 1978).

Microtubules elongate along the axis of motion in neutrophils during chemotaxis and shorten across the axis of motion (Anderson, et.al., 1982). Eutenuer and Schliwa (1985) have shown that actin in the lamellipod (leading edge) of polarized neutrophils associates with microtubules and stabilizes the centrosome/ microtubule complex. They also propose that that this connection constrains the cells ability to turn. The observations of Anderson, et. al., and Eutenuer and Schliwa can be explained within the framework of the "dynamic instability" hypothesis of Mitchison and

Kirchner (1984). These authors have studied the kinetics of microtubule assembly/ disassembly and propose that the tubulin within a cell is in a steady-state of association into and dissociation from microtubules. Different microtubules are lengthening and shortening, simultaneously. Those microtubules which are "capped" on one end can lengthen by addition of tubulin to the opposite (centrosomal) end. If polymerized actin in the lamellipod of a polarized neutrophil "caps" microtubules selectively then those microtubules will lengthen along the axis between the leading edge of the cell and the centrosome, while other microtubules dissassemble. However, Anderson et.al. (1982) also noted that microtubules in neutrophils are very labile and turnover rapidly. If a cytoplasmic structure imposes order on the moving cytoplasm, it must be stable enough to maintain orientation, not just establish it. In addition, "capping" of microtubules at the lamellipod end does not explain the observed lengthening of microtubules between the centrosome and the tail of the neutrophil during chemotaxis.

The intermediate filaments are the likely candidate for the maintenance of of orientation because of their stability. When the cell polarizes, in response to a ligand, occupied receptors move (are moved?) to the rear of the cell. If the intermediate filament network stabilises the rear of the cell by associating with the cell surface, through the occupied receptors, then an orientation may be maintained.

The "intermediate filament-membrane" connection may be stable, but the rear of the cell will still be flexible. In the absence of a drag force, contractile waves originating from different points near the front of the cell will not be resolved into a consistent vector of force along an established axis. Intermediate filaments have been shown to associate with microtubules through MAP-2 (Bloom & Valle, 1983), but such an association has not been demonstrated in neutrophils. If an intermediate filament-receptor complex associates with microtubules: 1) those microtubules may be "capped' and elongate into the rear of the cell,. 2) the rear of the cell will become less flexible and provide the extra stability to maintain an orientation. Finally, if this "extra stable" association is periodic, the characteristic time between turns would change, suggesting a mechanical distinction between chemotaxis and chemokinesis. Gruler, et al., (1984) have measured the characteristic times between turns in locomoting neutrophils and found them to be ˜30 sec. during chemokinesis and ˜75 sec. during chemotaxis.

The essential features of this model are illustrated in Fig 2.

6. Phagocytosis results in the removal of membrane from the cells' surface. Neutrophils pause after engulfing a particle, before they begin to move again. Presumably, this period is necesary for the replenishment of surface receptors. Phagocytosis can lead to maximal stimulation when the

cell encounters so many targets that the process of engulfing the particles, followed by phagosome-lysome fusion results in degranulation.

7. Since the same ligand-receptor interaction can produce motion or secretion, what constitutes maximum stimulation and degranulation is an important question in inflammation. The "receptor-cytoskeletal" connection suggests that the second messenger for secretion may be different from that needed to initiate locomotion. Jesaitis, et.al.(1984) showed that a transient ligand-receptor-cytoskeleton complex forms which increases the affinity of the receptor. If the receptor-cytoskeletal connection is disrupted with dihydro-cytochalasin D, the affinity of the receptors decreases and granular secretion is enhanced. This affinity-dependent difference in receptor function does not resolve the question of the second messenger for secretion. The timing of the release of second messengers may be important. The mechanisms of stimulus-secretion coupling have lag times of several seconds: esp. phosphoinositide metabolism (Dougherty, et.al. 1984). If a resting cell receives a "strong" stimulus, the formation of the high affinity complex may be blocked by the rapid binding of a large number of low affinity receptors and degranulation occurs. Sub-maximal stimulation does not interfer with the formation of the high-affinity complex and there is an almost instantaneous depolarization and release of membrane calcium which initiates motion. As the cell

encounters increasingly higher concentrations of ligand the receptors are down-regulated (negative cooperativity, Marasco, et.al., 1984) until the high-affinity, locomotion activating complex can no longer form and the cells degranulate. This self-modulation of receptor affinty may select which second messenger is activated.

8. Degranulation is a dead-end response becuase the massive fusion of granular membrane alters the cell surface and the cell cortex. When the cell degranulates, the viscosity of the actin-rich cortex must be alterd to permit fusion with the membrane. This often is accompained by the formation of invaginations from the membrane into the cell and the extension of pseudopods (Chandler, et. al., 1983). This disruption of the actin cortex and membrane seems to be irreversible since neutrophils are not immune to their own toxic products.

The proposed scheme can also explain such phenomena as "frustated phaocytosis". When a migrating cell encounters a region of the substrate which is the source of the ligand (eg., an adsorbed protein), the cell tries to engulf an infinitely large particle by spreading itself on the surface. It then degranulates onto the surface of this "particle".

DISEASES

This sequence can explain the observed motile defects associated with some disease states.

Chediak-Higashi Syndrome (CHS) is a genetic defect characterized by recurrent pyogenic infections, formation of giant lysosomal granules and defective chemotaxis (Gallin, 1980). Bacteriacidal capacity and random migration (chemokinesis) are normal (Clark, 1982). Neutrophils from CHS patients have greatly elevated levels of cAMP (Quie & Cates, 1978). In our model, the failure to maintain directed locomotion (step 3, failure to respond to a gradient) suggests a structural defect involving microtubules and intermediate filaments; an effect on neutrophil which is similar to that observed after treatment with colchicine. Ultrastructural studies of neutrohphils from these patients revealed a greatly reduced number of centriole- associated microtubules (Roberts & Gallin, 1983). When CHS patients were treated with ascorbic acid (which reduces intracellular cAMP), a significant restoration of chemotactic responsiveness was observed, due to either a restoration of a normal cAMP/cGMP ratio (Oliver, 1976) or a direct stabilization of microtubules (Boxer, et. al., 1979).

Actin dysfunction syndrome is characterised by defective chemotaxis and phagocytosis, but enhanced lysosomal exocytosis. Actin from these patients will not polymerize <u>in vitro</u> and actin-binding protein is either defective or missing (Quie, 1983, Roberts & Gallin, 1983). These effcts are similar to the effects produced by cytochalasins. Our model could account for this by a defect at step 1 (the

receptor affinity). Defective actin polymerization prevents the formation of the high affinity receptor-cytoskelton complex and degranulation is induced by the ligand-receptor interaction. The decreased viscosity of the cell cortex facilitates granule-plasmalemma fusion.

Further studies

An interesting model system has been developed which involves the induction of fragmentation of neutrophils by heating (Keller & Bessis, 1975, Malawista, 1982). When neutrophils are heated to 45°C for 9 min., the leading lamellipod pulls away from the rear of the cell. This free lamellipod is referred to as a cytokinetoplast. It is capable of chemotaxis (Dyett et.al.,1985), but is devoid of granules, nuclei and microtubules (Malawista, et.al., 1982). This cytokinetoplast is stable at room temperature for up to 29 hrs due to the lack of granular oxidative enzymes (Malawista, et.al., 1985). The behavior of this system is also inconsistent with our model because our model requires a structure in the rear of the cell, which includes microtubules, to maintian orientation.

The findings of our studies and these new results suggest several avenues of future research:

1. Our hypothesis that regulation of locomotion is a mechanical process mediated by specific cellular structure requires direct evidence that microtubules and intermediate filaments are involved (Chapt. 4,6). Motion analysis of the

cytokinetoplast undergoing chemotaxis would provide such direct, unequivocal evidence, since alterations of persistence and orientation accuracy could be attributed to the structures which were missing from the cytokinetoplast.

2. The precise role of intracelluar calcium in neutrophils chemotaxis is under intense investigation (cf. Sklar, et.al., 1985, Slonczewski, Wilde & Zigmond, et.al., 1985, Sha'afi, et.al., 1986)). The subcellular distribution of calcium during chemotaxis and phagocytosis has been measured directly in individual neutrophils (Sawyer, Sullivan & Mandell, 1985) and the highest concentrations were found in the lamellipod and pseudopods. This intracellular gradient could be the signal for the switch from chemokinesis to chemotaxis (step 3). Similar direct measurements could be made on cells during chemokineis and on those stored cells which were found to orient at random in a gradient to determine if <u>local</u> changes in calcium are the second messenger for orientation.

3. Several kinds of studies of the effects of storage remain to be done:

a. the time course of the storage "lesion" may be determined by performing motion analysis on cells at regular intervals (eg., every 6 hrs.).

b. the time-course of the effects of storage on chemokinesis came be determined in a similar manner to test the part of our hypothesis which impliys that chemokinesis is not

significantly altered during storage.

This dissertation reports a series of studies which could be the beginning of an extensive investigation of the mechanics and mechanism of neutrophils chemotaxis.

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FIGURE LEGENDS

1. When an unstimulated neutrophils is exposed to a chemoattractant ligand, it may be activated by a ligand-receptor interaction. The first behavioral response is a change in shapoe and the initation of contractions in the cell cortex. The first decision point involves the presence of a gradient of chemoattractant. If there is no gradient present, the cell migrates with a speed, PI and CI which are characteristic of chemokinesis. If there is a gradient present, but the cell cannot respond to it the resulting locomotion is indentical to chemokinesis. If the cell can respond to the gradient, the cell migrates with a speed, PI and CI which are characteristic of chemotaxis.

If the source of the chemoattractant ligand is not located, locomotion will continue so long as the ligand-receptor interaction continues. If the source is located and is a particle, the cell will phagocytose it. If the cell is not "maximally" stimulated, it will continue to migrate. If the source is not a particle but a localized region of ligand, the cell will continue to migrate. If the concentration of ligand or the extent of phagocytosis (number or kind of particle) is "maximal", the cell will degranulate by releasing its primary lysosomes. The degranulated cell will stop migrating and cannot be reactivated.

2. During chemokinesis, the actin in the lamellipod "caps" the microtubules which elongate between the lamellipod and the centrosome. This level of stability produces some degree of directional persistence (PI = ~0.7), lower speed and no orientation. The intermediate filaments (IF) are not associated with microtubules.

The response to a gradient involves the association of the intermediate filaments with the microtubules which become "capped" and elongate into the rear of the cell. This level of stability produces higher speed, higher directional persistence (PI= ~0.9) and precise orientation (standard deviation = ~30^{o}).

Storage affects the IF-microtubule association and produces a shift to a "chemokinetic" response to a gradient.

FIGURE 1

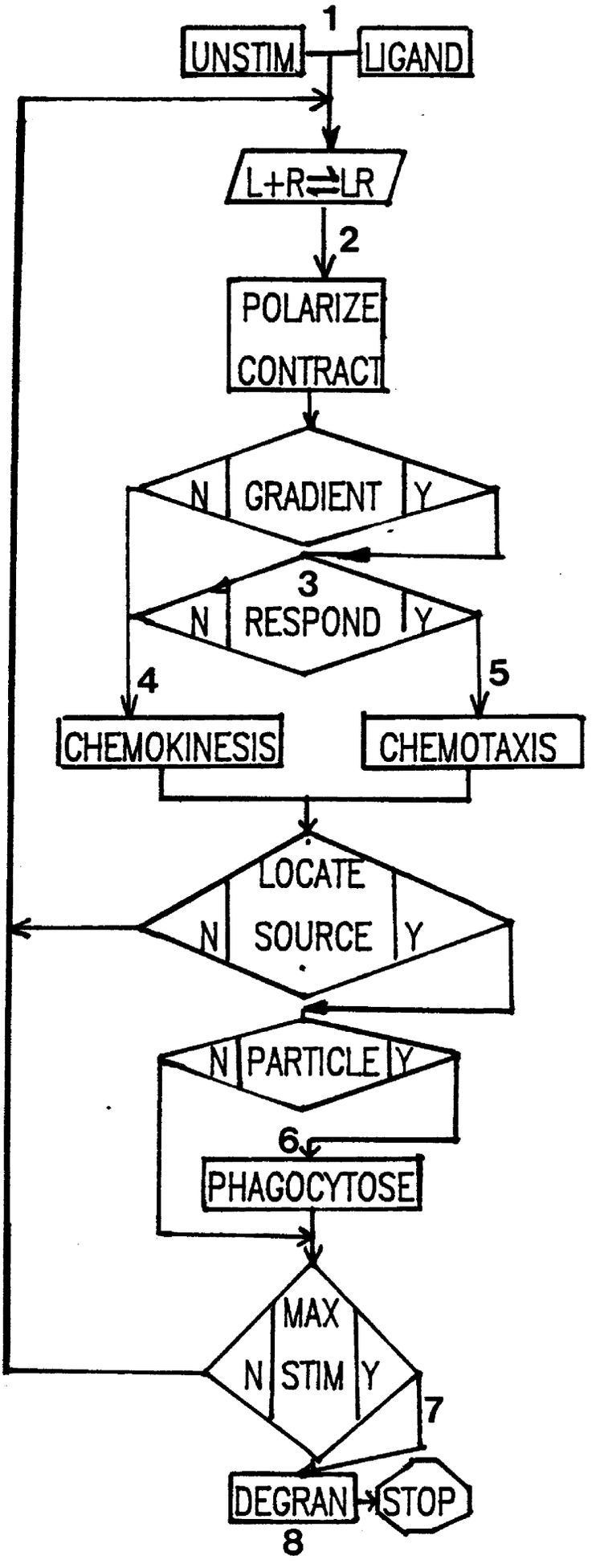
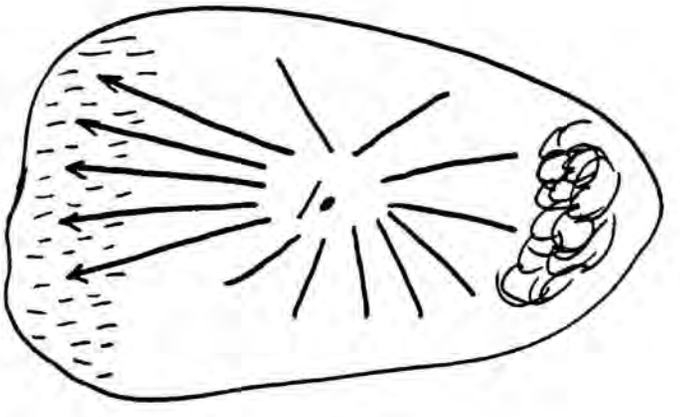
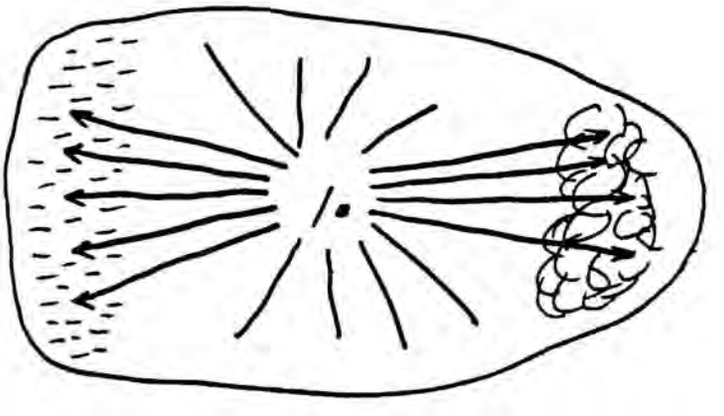

1
UNSTIM
LIGAND
L+R⇌LR
2
POLARIZE
CONTRACT
N
GRADIENT
Y
3
N
RESPOND
Y
4
5
CHEMOKINESIS
CHEMOTAXIS
LOCATE
N
SOURCE
Y
N
PARTICLE
Y
6
PHAGOCYTOSE
MAX
N
STIM
Y
7
DEGRAN
STOP
8

FIGURE 2

Hypothesis for the Structural
difference between chemokinesis (CKS)
and chemotaxis (CTX)



CKS



CTX

microfilaments, microtubules, intermediate filaments

"capped" microtubules (⟶)

centrosome (⁄•)